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14. ABSTRACT

In Year 3 of the project progress has been made toward our original Specific Aims (SAs) and broader central goal of elucidating the neuroprotective potential and mechanisms of purines implicated in the neurodegeneration of Parkinson's disease (PD). Published findings during Year 3 included our demonstrations that lowering urate in vivo using allopurinol could exacerbate neurotoxicity at the level of striatal dopamine (**SA2**); and that inosine a urate precursor could be protective in its own right against dopaminergic cell death in a cell culture model of PD(**SA3**). We also published methodological progress developing a new generation of more refined genetic probes of urate neurobiology. Recently we have obtained preliminary data identifying the Nrf2 antioxidant response pathway as an astrocytic mediator of urate's neuroprotective effects.Together our findings strengthen the rationale for pursuing purine targets as candidate neuroprotective strategies for PD, and have epidemiological and military, as well as translational significance.

15. SUBJECT TERMS

urate, tri-oxy-purine, synuclein, neuroprotection, neurotoxin, Parkinson's disease

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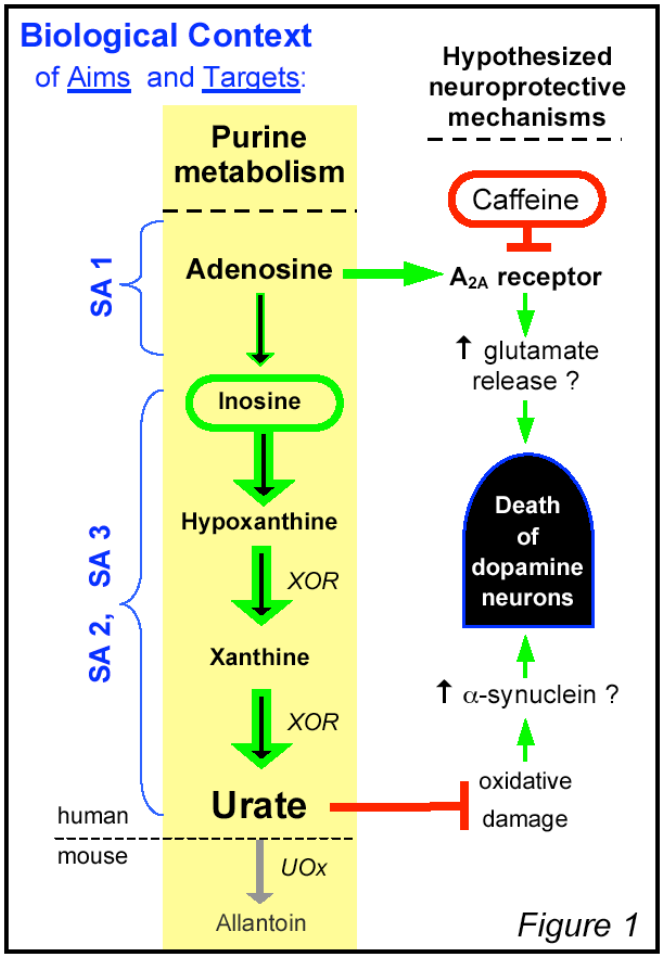
Appendices A-G – Year 3 Publications (2013-2014), *acknowledging DoD/W81XWH--1-0150*); see pg 5 for listing of Appendices/publications.

Introduction (unchanged from proposal SOW)

The overarching aim of the proposed work is to characterize the ***mechanisms and neuroprotective potential of purines linked to better outcomes in Parkinson's disease*** *(PD)*. We will pursue 3 Specific Aims (SAs) outlined in Section 3 below, and schematized in Figure 1 in the context of purine metabolism and dopaminergic neuron death. **SA1** seeks to determine the effects of the adenosine A_{2A} receptor antagonist caffeine as well as of neuronal A_{2A} receptor knockout (KO) in unilateral toxin models of PD. The potential role of excitotoxic glutatmate release will be investigated. **SA2** will assess the effects of the antioxidant urate (a.k.a. uric acid) on neurotoxicity *in vivo* using complementary pharmacologic and genetic approaches. Inosine, a therapeutically relevant urate precusor, will be tested along with genetic manipulations of urate metabolism, including global KO or conditional KO (cKO) of the *urate oxidase* (*UOx*) or *xanthine oxidoreductase* (*XOR*) genes. **SA3** will explore oxidative and α-synuclein mechanisms of urate protection in a neuronal cell culture models of PD. We propose to systematically pursue the following work on each SA with completion times indicated in brackets.



SA 1: **Mechanisms of protection by caffeine in toxin models of PD *in vivo***

Aim 1a: After establishing a 2,4-dichlorophenoxyacetic acid (2,4-D) model in mice [**Yr 1**], comparing the effects of systemic caffeine on 2,4-D *vs* 6-OHDA toxicities [**Yr 2**].

Aim 1b: Caffeine effects on toxin-induced glutamate release assessed by microdialysis [**Yr 3**].

Aim 1c: A_{2A} receptor dependence of caffeine effects on protection [**Yr 4**] and release [**Yr 5**].

SA 2: **Neruoprotection by urate in a unilateral toxin model of PD *in vivo*.**

Aim 2a: Determine *UOx* KO phenotype [**Yr 1**] and superimposed inosine effect in *UOx* KO [**Yr 2**].

Aim 2b: Localize the influence of increased urate on neurotoxicity using *UOx* cKO (Cre/*loxP* system) mice to elevate urate discretely in dopaminergic neurons [**Yr 3**] *vs* astrocytes [**Yr 4**].

Aim 2c: Assess for protection by *XOR* cKO (Cre/*loxP* system) with low urate in dopaminergic neurons [**Yr 4**] *vs* astrocytes [**Yr 5**], after completing floxed *XOR* mouse generation [**Yr 3**].

SA 3: **Mechanisms of protection by urate in toxin models of PD in neuronal cultures.**

Aim 3a: Determine inosine and urate effects on neurotoxicity and associated oxidative damage, MAPK pathway activation and α-synuclein expression [**Yr 1**].

Aim 3b: Effects of endogenous urate increase in *UOx* KO [**Yr 2**] and decrease in *XOR* KO [**Yr 4**].

Aim 3c: Effects of local urate increase in *UOx* cKO [**Yr 3**] and decrease in *XOR* cKO [**Yr 5**].

Body of the Report:

We report significant progress during Year 3 on the three of our original SAs, particularly SA 2 and 3 focused on exploring the role urate in PD models *in vivo* and in cell culture, respectively. Our progress is highlighted by and detailed in multiple publications (explicitly acknowledging support by DoD/NEPTR/W81XWH-11-1-0150) in Year 3, which are attached as Appendices A-G, listed as follows (with most relevant project **SA** indicated along with ***annotation*** of significance):

Appendix A: Kachroo A, Schwarzschild MA. (2014) Allopurinol reduces levels of urate and dopamine but not dopaminergic neurons in a dual pesticide model of Parkinson's disease. *Brain Res*. 1563:103-9. – **SA 2** [***Pharmacological study using a metabolic enzyme inhibitor to lower urate levels and explore the drug's effect in an environmentally relevant pesticide model of PD.***]

Appendix B: Cipriani S, Bakshi R, Schwarzschild MA. (2014) Protection by inosine in a cellular model of Parkinson's disease. *Neuroscience*. 274:242-9. – **SA 3 [*Inosine produced antioxidant and protective effects on dopaminergic cells, apparently and unexpectedly by a mechanism that does not require increased urate concentration. Nevertheless, the finding further supports inosine as a candidate for PD therapy.*]**

Appendix C: McFarland NR, Dimant H, Kibuuka L, Ebrahimi-Fakhari D, Desjardins CA, Danzer KM, Danzer M, Fan Z, Schwarzschild MA, Hirst W, McLean PJ. (2014) Chronic treatment with novel small molecule Hsp90 inhibitors rescues striatal dopamine levels but not α-synuclein-induced neuronal cell loss. *PLoS One*. 9(1):e86048. – **SA 3** [***This collaborative study characterized modulators of α-synuclein toxicity as a genetic model of PD.***]

Appendix D: Hung AY, Schwarzschild MA. (2014) Treatment of Parkinson's disease: what's in the non-dopaminergic pipeline? *Neurotherapeutics*. 11(1):34-46. – **SA 1** [***Review highlighting caffeine/adenosine A_{2A} translational prospects for PD.***]

Appendix E: McFarland NR, Burdett T, Desjardins CA, Frosch MP, Schwarzschild MA. (2013) postmortem brain levels of urate and precursors in Parkinson's disease and related disorders. *Neurodegener Dis*. 12(4):189-98. – **SA 2/3** [***This manuscript provides post-mortem evidence that low levels of nigrostriatal urate could contribute to the development of PD.***]

Appendix F: Bakshi R, Maguire M, Logan R, X Chen, Schwarzschild MA. (2013) Astroglia-dependent protective mechanisms of urate in a cellular model of Parkinson's disease. *Society for Neuroscience Annual Meeting* (San Diego, CA) Abstract # 714.09. – **SA 3** [***This presentation describes our recent data elucidating the mechanism of neuroprotection by urate.***]

Appendix G: Zuo F, Maguire M, Logan R, Xu Y, Chen X, Schwarzschild M. (2013) Urate oxidase knockout in mice by inducible *cre* and their CNS phenotype. *Society for Neuroscience Annual Meeting* (San Diego, CA) Abstract # 329.20. – **SA 2** [***This presentation highlights our technical progress in refining our genetic strategy of modeling urate elevation in humans by urate oxidase (UOx) mutation. Our development of these conditional UOx knockout mice is intended to circumvent developmental renal dysfunction that can confound interpretation of the global UOx knockout phenotype.***]

The above together with our previous papers listed in the prior project annual reports total at least 20 project publications directly citing W81XWH-11-1-0150.

Key translational/clinical trial publication during the reporting, which is directly supported by the findings published under DoD/NEPTR/W81XWH-11-1-0150:

The Parkinson Study Group SURE-PD Investigators, Schwarzschild MA, Ascherio A, Beal MF, Cudkowicz ME, Curhan GC, Hare JM, Hooper DC, Kieburtz KD, Macklin EA, Oakes D, Rudolph A, Shoulson I, Tennis MK, Espay AJ, Gartner M, Hung A, Bwala G, Lenehan R, Encarnacion E, Ainslie M, Castillo R, Togasaki D, Barles G, Friedman JH, Niles L, Carter JH, Murray M, Goetz CG, Jaglin J, Ahmed A, Russell DS, Cotto C, Goudreau JL, Russell D, Parashos SA, Ede P, Saint-Hilaire MH, Thomas CA, James R, Stacy MA, Johnson J, Gauger L, Antonelle de Marcaida J, Thurlow S, Isaacson SH, Carvajal L, Rao J, Cook M, Hope-Porche C, McClurg L, Grasso DL, Logan R, Orme C, Ross T, Brocht AF, Constantinescu R, Sharma S, Venuto C, Weber J, Eaton K. Inosine to Increase Serum and Cerebrospinal Fluid Urate in Parkinson Disease: A Randomized Clinical Trial. *JAMA Neurol*. 2014 Feb;71(2):141-50. [***Our successful phase II trial of inosine as urate-elevating strategy was supported by the project's laboratory findings and are facilitating our ongoing pursuit of phase III trial development – currently under review at NIH/NINDS***.]

In addition to completing the above publications during the current reporting period, our experimental activity over the last quarter (Apr-Jul 2014) since our last written (quarterly) report on the project is includes the following as examples of our further as yet unpublished findings during the current reporting period.

SA 2: Neruoprotection by urate in a unilateral toxin model of PD in vivo.

Aim 2a: Determine UOx KO phenotype [Yr 1]; superimposed inosine effect in UOx KO [Yr 2].

Aim 2b: Localize the influence of increased urate on neurotoxicity using UOx cKO (Cre/loxP system) mice to elevate urate discretely in dopaminergic neurons [Yr 3] vs astrocytes [Yr 4].

Despite demonstrated protective effect in 6-OHDA model of PD, *UOx* global KO mice show systemic toxicity of hyperuricemia that could confound further mechanistic study of urate in PD animal models. Our Y3 effort therefore focused on creation and characterization of conditional *UOx* KO mice by crossing floxed *UOx* line with a transgenic inducible (tamoxifen-responsive) *cre* line. After systemically exposing to the estrogen receptor ligand tamoxifen (i.p.), adult mice homozygous for the floxed *UOx*gene ($UOx^{fl/fl}$) and hemizygous for the transgene (*cre-ERT2*) successfully display recombination and partial disruption of the floxed *UOx* gene, with resultant reduction in hepatic UOx protein and increase in serum urate levels. The overall ~70% urate increase represents a urate-elevation phenotype intermediate between the homozygous constitutive *UOx* KO (10-fold serum urate elevation above WT) and heterozygote urate constitutive *UOx* KO (no detectable elevation above WT), and as expected, is not associated with obvious general toxicity. *UOx* cKO mice therefore are well suited for testing pharmacological effects of inosine at doses that will further elevate urate, as it does in (*UOx*-deficient) humans.

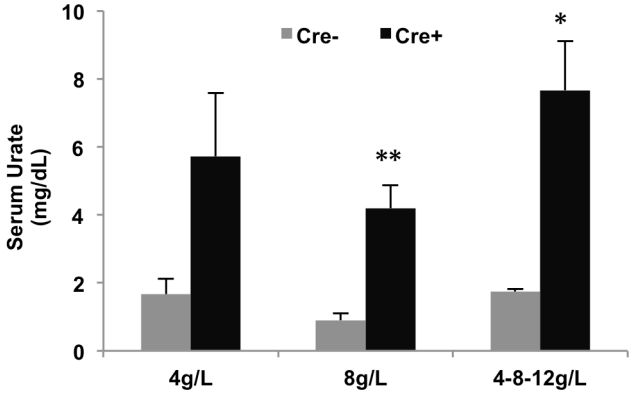


Fig. A: Serum urate in inosine-treated UO_X cKO mice. Serum urate were determined by HPLC. Adult male, n=5 and 4, CRE- and CRE+. **p*<0.05, ***p*<0.01 vs CRE-.

We conducted a pilot dose-effect experiment. Seven days after completion of tamoxifen injection regimen (100mg/kg for 2 x 5days), we treated male *UOx* fl/fl *Cre* mice with inosine in drinking water. As shown in Fig. A, inosine at 4g/L induced a strong trend for increased serum urate after 1 week. The difference between Cre+ and Cre- became statistically very significant at 4 weeks after 8g/L in drinking water. Increasing dose of inosine from 4g (for 3days) to 8 (for 4days), then to 12g/L (for 7 days) caused systemic toxicity and death. Inosine did not appear to have effect on serum urate levels in control *UOx* fl/fl *Cre*- mice. Striatal urate is currently pending HPLC measurement. If significant difference in striatal urate is confirmed, we will pursue neuroprotection of inosine in *UOx* cKO in models of PD using tolerable and effective 8g/L of inosine.

SA #3: Protection by urate in cellular models of PD: anti-oxidant and a-synuclein mechanisms.

1. Protection of dopaminergic cells by urate requires its accumulation in astrocytes.

We have recently confirmed protective properties of urate in cellular models of PD. To date these models have been based on spontaneous cell death or that produced by oxidative and mitochondrial toxins, including H2O2, rotenone, MPP+, 6-hydroxydopamine (6-OHDA), glutamate and iron ions. In our recent studies, we also found that urate produced much of its protective effect indirectly via astrocytes (see Appendices E and F). They in turn release a potent neuroprotective factor, which differs from urate because incubation of medium conditioned by urate-treated astrocytes with commercially obtained urate oxidase (UOx) eliminates urate but not the protective influence.

To further characterize the mechanisms underlying the astrocyte-dependence of urate's neuroprotection in cellular models of PD, we have employed complementary biochemical techniques and targeted screens to identify the putative protective factor(s) released by urate-stimulated astrocytes. The MES 23.5 dopaminergic cell line, which is hybrid of murine neuroblastoma-glioma N18TG2 cells with rat mesencephalic neurons is used to assay the protective effect of urate or of conditioned medium from urate-treated astrocytes. We treated enriched astroglial cultures with varying concentrations of urate, or vehicle. Twenty-four hours later conditioned media is collected and immediately used for the following experiments or stored at -20^{o}C for later experiments. The MES 23.5 cells are pre-treated with increasing proportions of conditioned medium and then exposed to toxins. For cell viability evaluation, as shown in Figure 1A, Annexin–V–FITC (BD-Pharmingen) and propidium iodide (PI, Sigma) staining is followed by flow cytometry analysis using FACScan (BD Biosciences). Urate treated conditioned medium from astrocytes is significantly more protective against H_2O_2 induced MES 23.5 cell death (Figure 1).

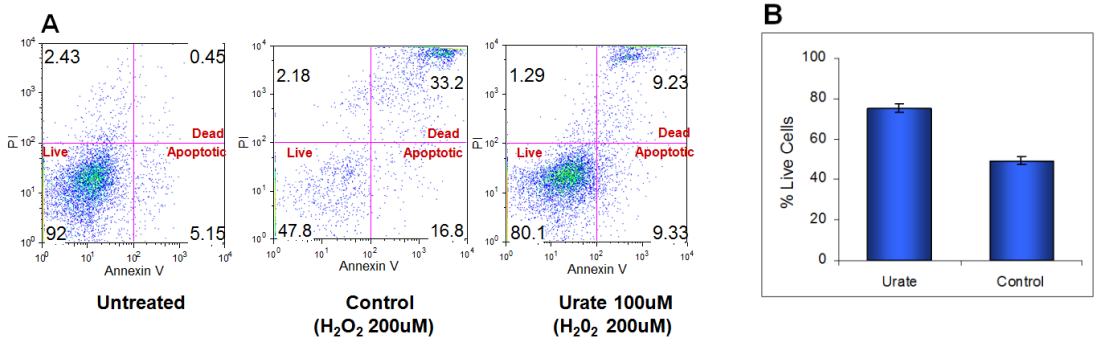


Figure 1. *Urate protects MES 23.5 cells from H_2O_2 induced cell death via conditioned medium from urate-treated astrocytes. (A) FACS analysis showing cell viability using PI/Annexin V staining. Percentages of PI+/Annexin V+ (necrotic), PI-/Annexin V+ (apoptotic) and PI−/Annexin V− (vital) staining is shown in MES 23.5 cells treated with conditioned medium from control or urate-treated astrocytes followed by H_2O_2 exposure. (B) Graphical representation of number of live cells in urate treated conditioned medium compared to vehicle control (n=3).*

2. ***Urate treatment causes release of glutathione (GSH) from astrocytes.***

Since we have shown a significant protective effect of conditioned medium from urate-treated astrocytes on MES 23.5 cell viability in presence of 200 uM H_2O_2, we have initiated

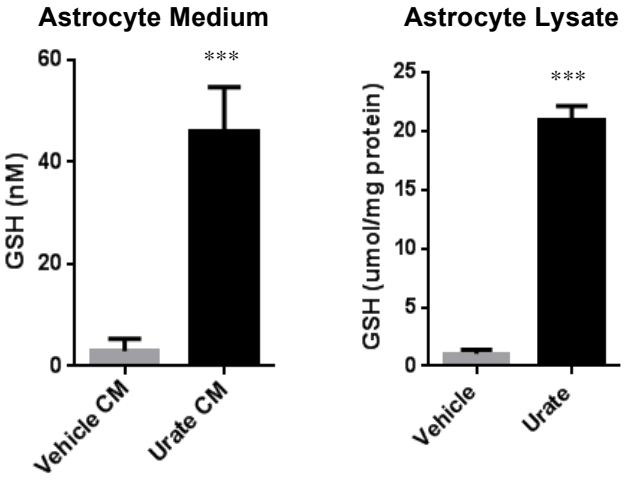


Figure 2. Marked elevation of GSH content in conditioned medium (CM) (**A**) and cell lysates (**B**) from urate- (versus vehicle-) treated astrocyte using GSH immunoassay (Abcam ELISA).*** denotes p value < 0.001; (n=4).

targeted screens to identify the putative neuroprotective factor in the conditioned medium from urate-treated (versus untreated) astrocyte using commercially available ELISA-based assay kits. We have assayed for prespecified established neuroprotective factors including glial-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), interleukin 6 (IL6) and glutathione (GSH). We identified GSH) as a primary candidate for the putative neuroprotective factor that is released from urate-treated astrocytes because it was the only one of the screened candidate neuroprotectants to be detected at a higher concentration in conditioned medium from urate-treated (compared to control) astrocytes (Fig 2).

3. Urate treatment upregulates GSH synthesis in astrocytes possibly by activating Nrf2 signaling pathway.

To further investigate the molecular mechanisms of urate-induced, astrocyte-dependent neuroprotection, we measured total GSH content in the astrocytes. The total GSH content in astrocytes was significantly increased in urate treated cells (Figure 2).

Since GSH biosynthesis is induced by Nrf2 activation, we assessed the regulation of Nrf2 target genes in urate-treated astrocytes by quantitative PCR (qPCR) assay. Total RNA from urate-treated and control astrocytes were extracted and reverse transcribed into cDNA (Invitrogen) and used for the qPCR assay. B-actin and Lamin were used as internal controls. Expression levels of γ-glutamyl cysteine synthetase modifier (GCLM) a rate limiting enzyme of GSH synthesis and Heme- Oxygenase 1 (HO-1) enzyme that catalyzes the degradation of heme was increased (Figure 3). These two enzymes are prominent target genes of Nrf2 transcription factor. We are currently in process of analyzing protein levels of Nrf2 and other target genes in astrocytes to validate these results.

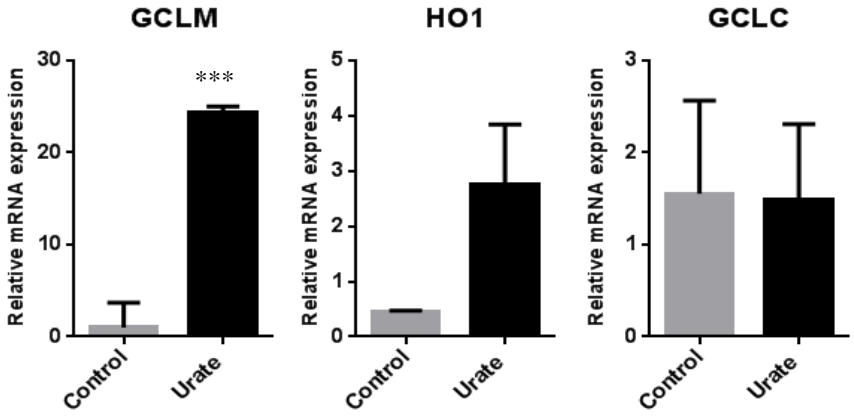


Figure 3. Urate-induced Nrf2 target gene expression. Quantitative PCR analysis in urate treated vs. untreated (control) cells of select Nrf2 target genes. Mean ± SEM are shown (N = 3). GCLC (Glutamate-Cysteine Ligase, Catalytic subunit) and is the non-regulated counterpart to GCLM. ***denotes p value < 0.001.

4. Inhibition of GSH synthesis diminishes urate's neuroprotective effect.

We are currently pursuing validation of GSH as a candidate mediator of urate's neuroprotective effect by studying the effect of inhibiting GSH synthesis in conditioned media from urate-treated astrocytes in order to definitively assess its role in neuroprotection conveyed by the conditioned medium. We block GSH function in the conditioned media by targeting its synthesis by neuronal cells. **L-Buthionine-sulfoximine (BSO)** inhibits GSH synthesis thus reducing cellular GSH level (Figure 4).

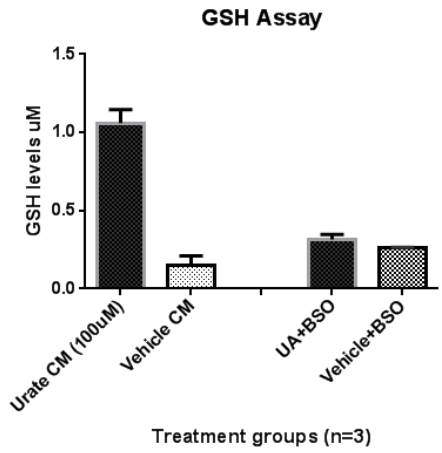


Figure 4. Total GSH content was significantly increased in urate-treated (versus untreated) astrocyte cell lysates. L-Buthionine-sulfoximine (BSO) inhibits GSH synthesis thus reducing cellular GSH levels.

We performed cell viability experiments (as described above) in MES 23.5 dopaminergic cells treated with Urate or vehicle conditioned media in presence of BSO. Neuroprotection by urate conditioned media was significantly reduced in presence of BSO which indicates that presence of GSH in the conditioned media is critical for urate's protective effect (Fig 5).

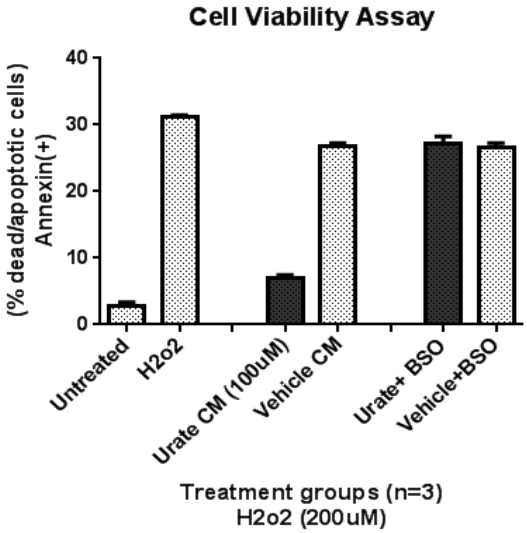


Figure 5. Cell Viability was significantly reduced in presence of BSO in MES 23.5 dopaminergic cells treated with urate conditioned media.

5. Nrf2-mediated neuroprotection by urate.

The neuroprotective effects of urate do not seem to rely on its long-established direct antioxidant properties. Instead urate appears to act through astrocytes by engaging an antioxidant signaling system orchestrated by Nrf2 ('Nuclear factor erythroid 2-related factor 2). As we have shown some key Nrf2 target genes are upregulated in primary astrocyte cultures treated with urate. Our group recently characterized the *UOx* cKO mice which have elevated urate levels in brain and are resistant to 6-OHDA toxicity. To assess the expression of key Nrf2 target genes in urate-elevating conditional *UOx* KO mice, we ran western blots on the striatal tissue of *UOx* cKO mice compared to the Cre- controls. Protein levels of *NQO1* and *GCLM* were significantly increased in the *UOx c*KO mice, indicating elevated Nrf2 signaling in these mice (Fig.6). We are currently pursuing Nrf2 mediated mechanisms of urate's neuroprotection in cellular and animal models of PD.

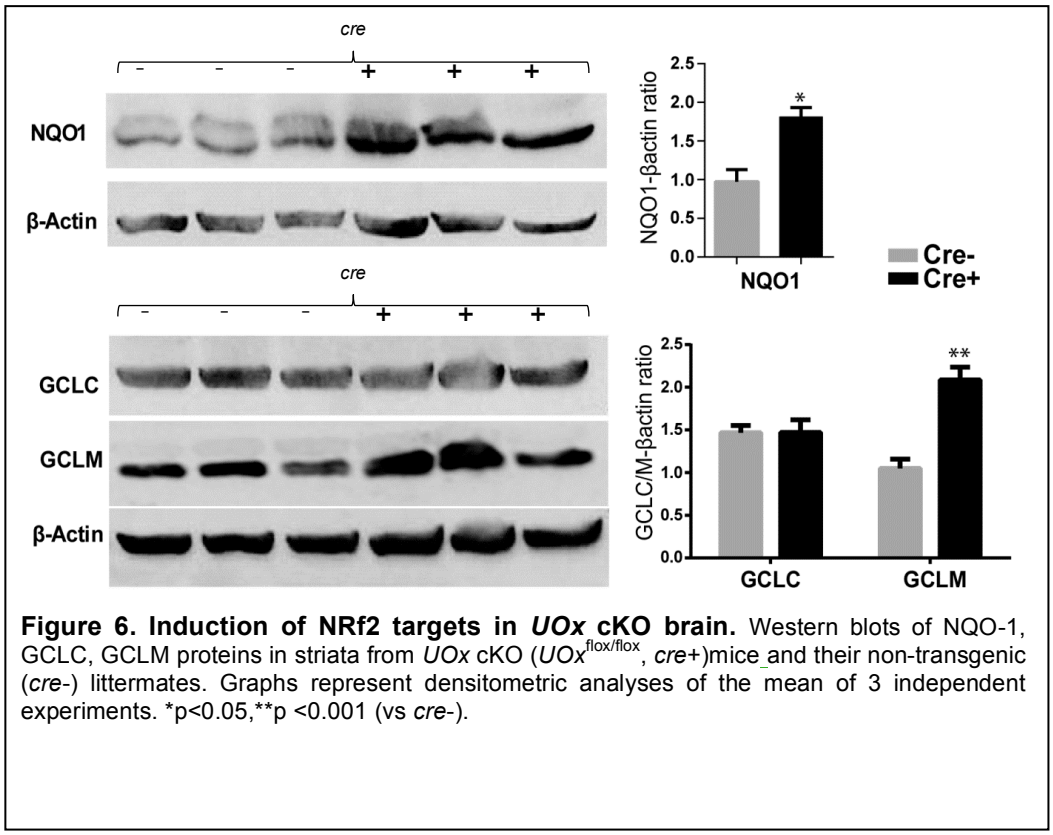


Figure 6. Induction of NRf2 targets in *UOx* cKO brain. Western blots of NQO-1, GCLC, GCLM proteins in striata from *UOx* cKO ($UOx^{flox/flox}$, *cre*+)mice and their non-transgenic (*cre*-) littermates. Graphs represent densitometric analyses of the mean of 3 independent experiments. *p<0.05,**p <0.001 (vs *cre*-).

Key Research Accomplishments (Year 3)

- Publications providing further preclinical evidence that inosine (a purine precursor of urate) currently in clinical development may produce protective effects of dopaminergic cells (Appendix B), and that urate itself may be protective against loss of striatal dopamine in models of PD (Appendix A).
- Characterization of next generation of genetic probes of urate function has demonstrated urate elevation in controlled (inducible) conditional knockout of the *urate oxidase* (*UOx*) gene (see unpublished progress above under SA 2), and in a *Glut9* urate transporter knockout.

- Gained evidence that the astroglial Nrf2 antioxidant response system is activated in by urate.

Reportable Outcomes

In Year 3 of the project we have published 9 manuscripts reflecting progress on the project (and in all cases explicitly acknowledging grant support from the DoD/NEPTR/W81XWH-11-1-0150) as follows, with relevant SA's noted in **bold**:

Cipriani S, Bakshi R, Schwarzschild MA. Protection by inosine in a cellular model of Parkinson's disease. *Neuroscience*. 2014 Aug 22;274:242-9. **– SA 3** (**Appendix A**)

Kachroo A, Schwarzschild MA. Allopurinol reduces levels of urate and dopamine but not dopaminergic neurons in a dual pesticide model of Parkinson's disease. *Brain Res*. 2014 May 14;1563:103-9. **– SA 3** (**Appendix A**)

McFarland NR, Dimant H, Kibuuka L, Ebrahimi-Fakhari D, Desjardins CA, Danzer KM, Danzer M, Fan Z, Schwarzschild MA, Hirst W, McLean PJ. Chronic treatment with novel small molecule Hsp90 inhibitors rescues striatal dopamine levels but not α-synuclein-induced neuronal cell loss. *PLoS One*. 2014 Jan 20;9(1):e86048.

Hung AY, Schwarzschild MA. Treatment of Parkinson's disease: what's in the non-dopaminergic pipeline? *Neurotherapeutics*. 2014 Jan;11(1):34-46

McFarland NR, Burdett T, Desjardins CA, Frosch MP, Schwarzschild MA. postmortem brain levels of urate and precursors in Parkinson's disease and related disorders. *Neurodegener Dis*. 2013;12(4):189-98.

Bakshi R, Maguire M, Logan R, X Chen, Schwarzschild MA. (2013) Astroglia-dependent protective mechanisms of urate in a cellular model of Parkinson's disease. *Society for Neuroscience Annual Meeting* (San Diego, CA) Abstract # 714.09.

Zuo F, Maguire M, Logan R, Xu Y, Chen X, Schwarzschild M. (2013) Urate oxidase knockout in mice by inducible *cre* and their CNS phenotype. *Society for Neuroscience Annual Meeting* (San Diego, CA) Abstract # 329.20.

Presentations (given by PI; *acknowledging W81XWH--1-0150 / NETPR / DoD*) included:

- September 2013 – (Stockholm, Sweden) Corticobasal Degeneration Solutions Symposium/ Karolinska Institutet Seminars. 'Molecular epidemiology of parkinsonism and its rapid translation to clinical trial"' *[invited seminar]*
- September 2013 – (Ft, Detrick, MD) ''Targeting Purine Therapeutics for Parkinson's: From caffeine to adenosine to urate"' US Dept. of Defense, TATRC/NETPR programs. *[invited seminar]*

- October 2013 – (New York, NY) "Inosine for Parkinson's Disease: Safety and Trial Design Optimization" The Michael J. Fox Foundation for Parkinson's Research and the New York Academy of Sciences; Seventh Annual Parkinson's Disease Therapeutics Conference () *[invited speaker]*
- February 2014 – (Las Vegas) Parkinson Study Group course on PD trials and therapeutics progress – speaker, "Neuroprotection in Parkinson's: Where do we stand?".*[speaker]*

Funding Applied for Based on the Work Supported by this Award includes:

NIH (NINDS) 1R01NS091493-01 (Schwarzschild, PI) 2015-2020 " Pursuing Epidemiological Clues to Neuroprotective Therapy for Parkinson's Disease" [pending].

NIH (NINDS) R21 in response to FOA PA-11-261, "NIH Exploratory /Developmental Research Grant Program" (new application; Schwarzschild and DK Simon, dual-PIs) 2013-2015 "Role of urate in protecting mitochondrial function in the brain" [Funded].

Michael J. Fox Foundation (LRRK2 Cohort Consortium) "*Purine Biomarkers of LRRK2 PD*" (Schwarzschild, PI) 2014-2016 [Funded].

Conclusions/Plans/Signficance

In the third year of the project we have made substantial progress toward the original SA's as documented in multiple research manuscripts generated (appendices). The results help establish that multiple purines (adenosine antagonists, inosine and urate) can confer neuroprotection in mouse models of Parkinson's disease. They provide a solid foundation on which to build our subsequent experiments, including those outlined in the SOW.

Plans – Major plans in Yr 4 include characterizing the PD toxin/genetic model phenotype of our newly characterized inducible/conditional (post-natal) knockout (KO) of *UOx*. This conditional KO obviates the confounding developmental phenotype of the constitutive *UOx* knockout (SA2). We will seek to confirm the determine the role of Nrf2 as an astrocytic mediator of urate's neuroprotective effect (SA3).

Significance -- Our characterization of the roles of these purines in mouse models of PD neurodegeneration through this preclinical project remains well positioned to inform and potentially accelerate the initiation of phase III clinical trials of neuroprotective candidates for the disease. Supported by our earlier progress on caffeine and adenosine (SA 1), human studies are under way investigating adenosine- as well as urate-targeted strategies in patients with PD. Recently caffeine itself (http://clinicaltrials.gov/show/NCT01738178) as well as more specific antagonism of the adenosine A_{2A} receptor (http://clinicaltrials.gov/show/NCT01968031) has entered clinical development in PD trials designed to assess disease-modifying effects. Similarly our own clinical development of inosine as a urate precursor targeted as a candidate neuroprotective strategy has reported results of phase 2 testing (http://clinicaltrials.gov/ct2/show/NCT00833690).

In addition to its high translational impact, our exploration of purines in preclinical models of PD has substantial epidemiological and military significance. The mechanistic insights pursued under this project reflect a prototypic interaction between putative environmental protectants (e.g., caffeine, urate) and toxins.

Appendix A

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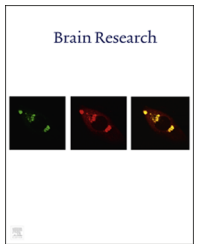
Brain Research



Research Report

Allopurinol reduces levels of urate and dopamine but not dopaminergic neurons in a dual pesticide model of Parkinson's disease

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ABSTRACT

Robust epidemiological data link higher levels of the antioxidant urate to a reduced risk of developing Parkinson's disease (PD) and to a slower rate of its progression. Allopurinol, an inhibitor of xanthine oxidoreductase (XOR), blocks the oxidation of xanthine to urate. The present study sought to determine whether lowering levels of urate using allopurinol results in exacerbated neurotoxicity in a dual pesticide mouse model of PD. Although oral allopurinol reduced serum and striatal urate levels 4-fold and 1.3-fold, respectively, it did not alter the multiple motor deficits induced by chronic (7 week) intermittent (biweekly) exposure to intraperitoneal Paraquat (PQ) plus Maneb (MB). However, striatal dopamine content, which was unaffected after either allopurinol or chronic pesticide exposure alone, was significantly reduced by 22% in mice exposed to the combination. Stereological assessment showed that the numbers of dopaminergic nigral neurons were significantly reduced by 29% and the tyrosine hydroxylase (TH) negative neurons unaffected after PQ+MB treatments. This reduction in TH-positive neurons was not affected by allopurinol treatment. Of note, despite the expectation of exacerbated oxidative damage due to the reduction in urate, protein carbonyl levels, a marker of oxidative damage, were actually reduced in the presence of allopurinol. Overall, allopurinol lowered urate levels but did not exacerbate dopaminergic neuron degeneration, findings suggesting that basal levels of urate in mice do not appreciably protect against oxidative damage and neurotoxicity in the PQ+MB model of PD, and/or that allopurinol produces an antioxidant benefit offsetting its detrimental urate-lowering effect.

1. Introduction

Epidemiological studies have identified both positive and negative risk factors for the incidence of Parkinson's disease (PD). Amongst environmental positive risk factors, pesticide exposures have been linked to an increased risk in developing PD, with Paraquat (PQ) and Maneb (MB) (Costello et al., 2009) among those implicated. Negative risk factors for PD include purine-based compounds, urate and caffeine. In fact, robust epidemiological data have linked higher levels of urate to

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a reduced risk of developing PD (Weisskopf et al., 2007; Chen et al., 2009) and of its progression (Schwarzschild et al., 2008; Ascherio et al., 2009). Urate accounts for most of the antioxidant capacity in human plasma (Yeum et al., 2004); with its antioxidant properties as powerful as those of ascorbic acid (Ames et al., 1981). Urate has been shown to specifically confer protection in cellular (Jones et al., 2000; Duan et al., 2002; Guerreiro et al., 2009) as well as in animal models of disease such as multiple sclerosis (Scott et al., 2002), stroke (Yu et al., 1998) and PD (Wang et al., 2010).

While it has been proposed that higher serum urate levels may be of selective advantage in the evolution of the hominids because of its antioxidant effects; hyperuricemia is associated with multiple diseases in humans and points to the deleterious effects of high concentrations of urate. Current approved pharmacological approaches to lower urate levels in patients with gout rely on allopurinol to reduce urate production (Bieber and Terkeltaub, 2004; Pea, 2005). Allopurinol, an inhibitor of xanthine oxidoreductase (XOR) blocks the successive oxidations of hypoxanthine to xanthine, and xanthine to urate. The enzyme XOR is widely distributed throughout various organs including the liver, heart, lung, brain as well as plasma and can exist in either one of 2 forms, xanthine dehydrogenase (XDH), predominating in healthy tissues or xanthine oxidase (XO) which plays an important role in injured cells and tissues (Harrison, 2002). Both forms are interconvertible with one another, with the XO subtype causing reduction of molecular oxygen leading to generation of reactive oxygen species (Berry and Hare, 2004).

The present study sought to determine whether pharmacologically lowering urate levels in mice using chronic allopurinol treatment alters the pesticide-induced neurotoxic phenotype in an environmental toxin model of PD.

2. Results

2.1. *Allopurinol and not PQ+MB significantly attenuates serum and striatal urate levels*

Serum urate levels of mice exposed to allopurinol in the drinking water were significantly decreased by approximately 4-fold ($p<0.0001$) compared to their unexposed water-drinking counterparts. PQ+MB treatment had no effect (Fig. 1A). Striatal urate levels were found to be significantly reduced ($p=0.0024$) in allopurinol treated mice though only by 1.3-fold compared to non-allopurinol treated mice (Fig. 1B). Measurement of additional purines included in the purine metabolism pathway such as hypoxanthine and xanthine was consistent with those published by Enrico et al. (1997) with no demonstrated differences after allopurinol treatment. The data values (ng/mg tissue units) for striatal hypoxanthine levels for the groups: Tap water–Saline, Tap–PQ+MB, Allopurinol–Saline and Allopurinol–PQ+MB were 0.04±0.005; 0.04±0.004; 0.04±0.004; 0.04±0.002, respectively. For the same groups striatal xanthine levels were 0.10±0.02; 0.12±0.03; 0.11±0.01; 0.08±0.01. The data values (mg/dL units) for serum hypoxanthine levels for the groups: Tap water–Saline, Tap–PQ+MB, Allopurinol–Saline and Allopurinol–PQ+MB were 0.041±0.003; 0.042±0.004; 0.04±0.003; 0.06±0.02, respectively. For the same groups serum xanthine levels were 0.0179±0.0014; 0.014±0.0006; 0.02±0.0010; 0.01±0.001. The lack of a change in the levels of both hypoxanthine and xanthine may be due to the fact that allopurinol increases the conversion of hypoxanthine to inosinic acid and the inhibition of the rate of de novo purine biosynthesis.

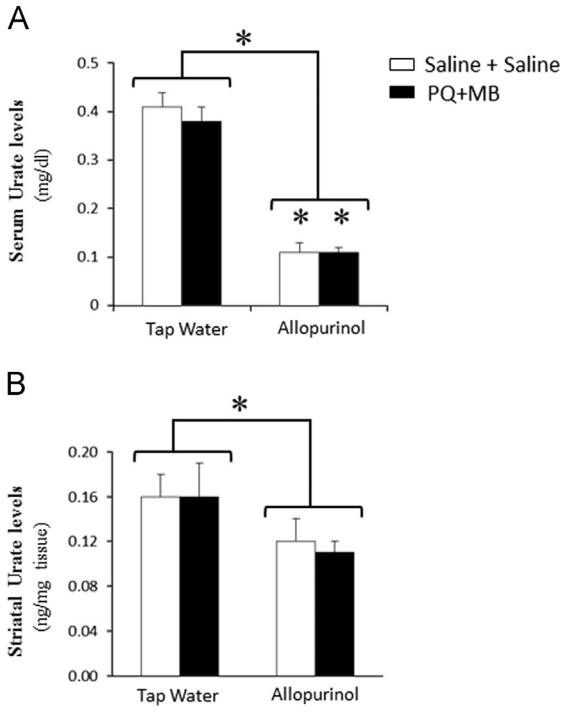

Fig. 1 – Evidence that Allopurinol and not PQ+MB significantly attenuates serum and striatal urate levels in mice. (A) Serum urate level, *$p<0.0001$ vs respective tap water treatment groups; *$p<0.0001$ (combined allopurinol vs combined tap water treatment groups); unpaired *t*-test. (B) Striatal urate level, *$p=0.024$ (comparison between combined allopurinol vs combined tap water treatment groups). Groups are: Tap water [Saline control ($n=8$); PQ+MB ($n=12$)]; Allopurinol [Saline control ($n=8$); PQ+MB ($n=11$)]. Combined treatment groups are: Tap water group ($n=20$); Allopurinol group [Saline control ($n=19$)].

2.2. *Allopurinol does not potentiate PQ+MB-induced motor dysfunction*

The pole test and the beam traversal task were used to detect any motor dysfunction that may reflect toxin-induced dopaminergic deficit. Specifically, MPTP-treated mice have been shown to display slower times in the descent time parameter of the pole test compared to controls, impairments reversed by L-dopa (Matsuura et al., 1997). In addition, Huntington disease knock-in mice displayed significant impairments in the pole test (Hickey et al., 2003), indicating it is a useful test for basal ganglia dysfunction. The beam traversal task is used to specifically assess fine-motor initiation, coordination and postural balance. Hwang et al. (2005) highlighted transgenic mouse models of PD as significantly slower in traversing

a narrow, raised beam as well as taking an increased number of steps, compared to wild-type control animals. These test parameters mimic the slower movements and shorter steps observed in PD patients. PQ+MB treated animals exposed to only water demonstrated a significant increase in duration required to descend the pole, reported as descent time (Fig. 2A); in addition to increased time required to cross the beam (beam latency) (Fig. 2B), as well as the number of steps required to do so (Fig. 2C). Allopurinol exposure did not seem to affect either of these measures.

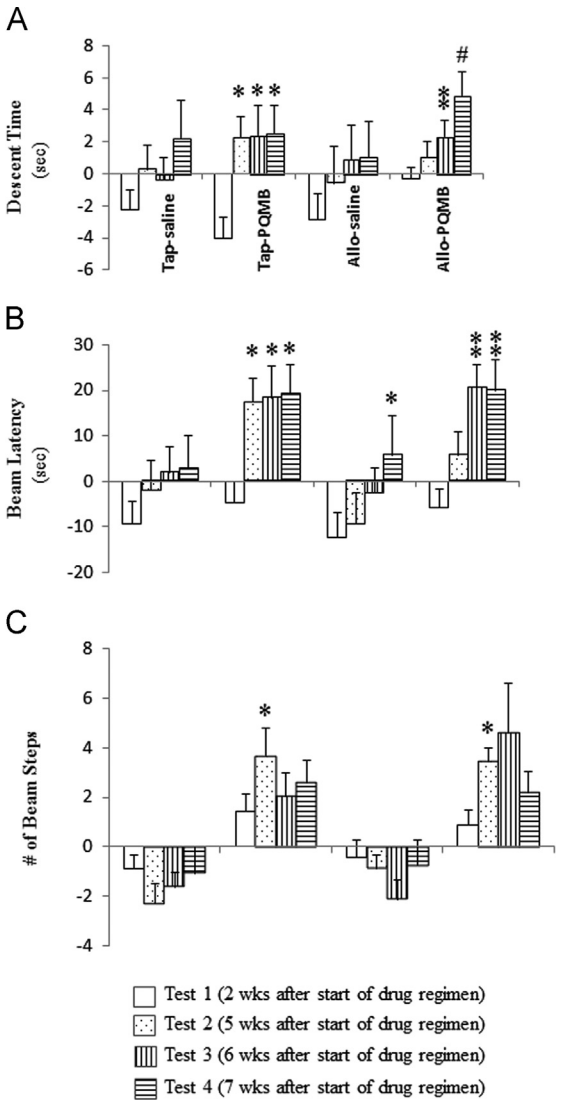

Fig. 2 – PQ+MB treatment significantly (A) increases time to descend the pole. *$p<0.01$, **$p<0.05$, #$p<0.001$ vs Testing 1; RMANOVA. (B) increases beam latency. *$p<0.01$, **$p<0.001$ vs Testing 1; RMANOVA. (C) increases number of beam steps. *$p<0.01$ vs Testing 1; RMANOVA. Data reported as Test–Baseline scores. Water [Saline control ($n=8$); PQ+MB ($n=12$)]; Allopurinol [Saline control ($n=8$); PQ+MB ($n=11$)]. Tests 1–4 correspond to behavioral assessments performed at 2, 5, 6 and 7 weeks respectively, after the start of the toxin injections.

2.3. *Allopurinol potentiates striatal DA loss in combination with PQ+MB exposure*

Chronic intermittent administration of PQ+MB on its own did not reduce striatal DA content, consistent with prior studies (Thiruchelvam et al., 2000a, 2000b). However, in the presence of urate-lowering allopurinol treatment PQ+MB significantly reduced striatal dopamine by approximately 22% (Fig. 3A).

2.4. *Allopurinol does not further exacerbate PQ+MB-induced loss of TH+ neuronal cells*

By contrast, PQ+MB treatment on its own significantly decreased the number of TH+ neurons in the substantia nigra pars compacta (SNpc) by approximately 28% compared to the corresponding saline-treated group (Fig. 3B). Allopurinol treatment did not alter dopaminergic neuron cell counts and did not potentiate pesticide-induced dopaminergic neuron cell loss. Direct assessment of TH− (negative) neurons confirmed the specificity of pesticide-induced neurotoxicity for dopaminergic neurons of the SNpc, as previously shown (Kachroo et al., 2010) (Fig. 3C). Allopurinol, either alone, or in

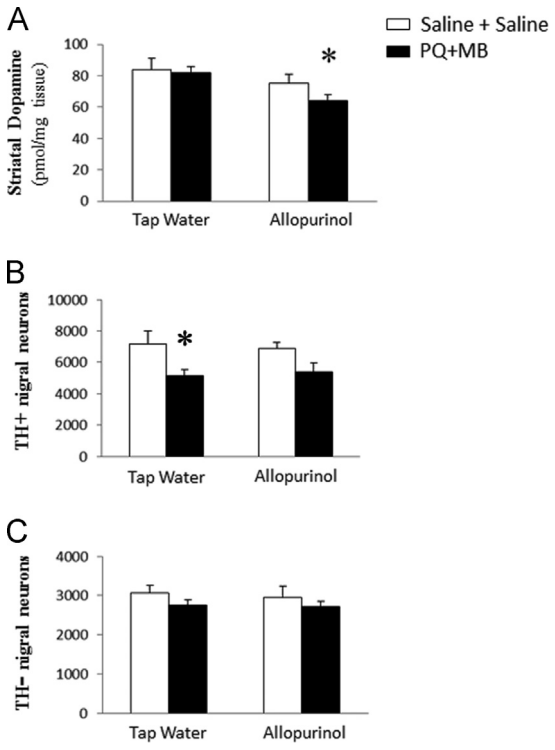

Fig. 3 – Allopurinol potentiates striatal DA loss in combination with PQ+MB exposure, but does not further exacerbate loss of total TH+ neuronal cells (bilateral) in the substantia nigra pars compacta (A) *$p=0.0038$ vs Water-PQ+MB group; unpaired t-test. (B) *$p=0.03$ vs Water–Saline group; unpaired t-test. (C) No significant differences within the Tap water group (Saline vs PQ+MB); Allopurinol group (Saline vs PQ+MB); between Tap water and Allopurinol groups (Saline vs Saline; PQ+MB vs PQ+MB). Water [Saline control ($n=8$); PQ+MB ($n=12$)]; Allopurinol [Saline control ($n=8$); PQ+MB ($n=11$)].

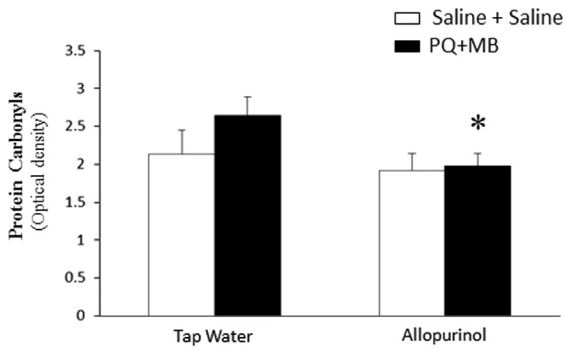

Fig. 4 – Allopurinol attenuates protein carbonyl levels in brains of PQ+MB-lesioned mice. *$p=0.043$ vs Water–PQ+MB; unpaired *t*-test. Water [Saline control ($n=8$); PQ+MB ($n=12$)]; Allopurinol [Saline control ($n=8$); PQ+MB ($n=11$)].

combination with PQ+MB had no effect on TH− (negative) neuron number.

2.5. *Allopurinol attenuates protein carbonyl levels in brains of PQ+MB-lesioned mice*

Measurement of oxidative damage assessed by the protein carbonyl assay showed a non-significant increase after PQ+MB treatment to levels that were significantly reduced (27%) by allopurinol (Fig. 4).

3. Discussion

These findings demonstrate that we can pharmacologically lower levels of urate both in serum and brain (striatum) of mice by chronic oral administration of allopurinol. Our findings are confirmatory with others showing significant attenuation of striatal urate levels after allopurinol treatment (Desole et al., 1995; Enrico et al., 1997; Miele et al., 1995). Under these experimental conditions we showed PQ+MB treatment itself did not directly affect urate levels.

Motor function, as assessed using the pole test and beam traversal task provided a behavioral measure of pesticide toxicity. This was unaffected by allopurinol alone and while PQ+MB treatment worsened motor performance after at least 6 weeks of toxin exposure (12 injections), urate-lowering allopurinol treatment did not affect these deficits. Previously, it has been shown that under certain conditions of *elevating* urate levels (after injections of intraperitoneal (i.p.) administered urate) in rats exposed to 6-hydroxydopamine, behavioral outputs such as locomotion scores and forepaw adjusting step test scores can be improved (Wang et al., 2010). Differences in rodent species, toxin used, extent/direction of urate change, and even our approach of *indirectly* targeting urate levels by inhibiting the enzyme XOR in our model may all contribute to the lack of a hypothesized behavioral effect.

Striatal dopamine content was shown to be unaffected after either allopurinol or chronic pesticide exposure but was significantly reduced in mice exposed to the combination. Based on prior literature, our data are consistent with allopurinol having no direct effect on striatal DA levels (Desole et al., 1995; Miele et al., 1995). The potentiated effect observed in the presence of a toxin may well be a result of allopurinol unmasking a PQ+MB-induced dopamine loss, possibly due to reduced endogenous antioxidant capacity resulting from lower striatal urate levels. In this setting PQ+MB may produce further increases in reactive oxygen species (ROS) and subsequent dopamine oxidation or dopaminergic nerve terminal injury.

In contrast to striatal dopamine levels, nigral dopaminergic cell counts were not reduced by allopurinol in the PQ+MB model of PD. Although such an exacerbation of neurotoxicity had been hypothesized based on the ability of allopurinol to lower levels of the putative antioxidant urate, we did not find a corresponding increase in oxidative damage markers in brain. Interestingly, brain levels of protein carbonyls were actually reduced by allopurinol in brains of mice treated with PQ+MB, suggesting a net antioxidant effect of allopurinol. Thus, at the level of nigral neuron survival, potentially deleterious urate-lowering effects of allopurinol may have been offset by antioxidant benefits. For example, in peripheral tissue, allopurinol or its metabolites can produce significant antioxidant effects on toxin-induced injury (Kitazawa et al., 1991; Knight et al., 2001), possibly PQ+MB via reduced XOR-driven H_2O_2 (as well as urate) generation. Why an antioxidant benefit of allopurinol would offset a detrimental effect of lower urate on nigral neuron numbers but not on striatal dopamine content is unclear, but may be related to the distinct anatomical and neurochemical nature of these of nigrostriatal neuron features. In any event, an alternative approach to testing urate reduction, such as may be achieved by increasing urate degradation (rather than by decreasing synthesis via allopurinol) could provide a simpler test of urate's role in models of PD. Finally, whether the synergistic toxicity of allopurinol and these pesticides on striatal dopamine levels (and the dissociation of allopurinol effects on nigral and striatal indices of dopaminergic neuron injury) were consistent across animal models of PD should be assessed in complementary, standard toxin (e.g., MPTP and 6-hydroxydopamine) and transgenic (e.g., α-synuclein) models of the disease.

4. Experimental procedures

4.1. *Drug administration*

Two-month-old male C57BL/6NCrl mice were obtained from Charles River Laboratories; Wilmington, MA and housed under a 12:12 h light:dark cycle. Food and water were provided ad libitum. All experiments were performed in accordance with Massachusetts General Hospital and NIH guidelines on the ethical use of animals, with adequate measures taken to minimize pain and discomfort. Prior to the start of the experiment, mice were either continued on water or placed on allopurinol dissolved in water for one month before exposure to intraperitoneal injections of saline or 10 mg/kg PQ (1,1′-dimethyl-4,4′-bipyridinium) dichloride hydrate followed immediately by 30 mg/kg MB (manganese bisethylenedithiocarbamate) in a volume of 10 ml/kg body

weight. Allopurinol was administered at a dosage of 150 mg/L and bottles replaced weekly. PQ and MB both obtained from Sigma were dissolved separately in saline on the day of administration and injected on the opposite sides (lower quadrants) of animal's abdomen. Mice were treated chronically (twice weekly for 7 weeks) in the following randomly assigned initial groups: Water [Saline control ($n=8$); PQ+MB ($n=18$)]; Allopurinol [Saline control ($n=8$); PQ+MB ($n=18$)]. Mortality rates of 33% and 39% in the toxin-treated mice on regular and allopurinol-treated water, respectively, resulted in final group numbers of 12 and 11. Body weights were obtained twice a week prior to injection during the course of the experiment. Drug treatments did not affect body weight (DNS). Mice were continued on allopurinol or water until their sacrifice one week after the last PQ+MB or Saline+Saline injection.

4.2. *Behavioral testing*

Behavioral testing involved mice being exposed to the pole test and the beam traversal task (Kachroo and Schwarzschild, 2012). Baseline values for individual mice on these tests were taken prior to, and during, allopurinol treatment but before toxin exposure. No behavioral differences were observed between either time-point. The average of both baselines was used to normalize subsequent assay values and thus reduce their variability. Mice were tested at 4 time-points referred to as Tests 1–4 which correspond to behavioral assessments at 2, 5, 6 and 7 weeks respectively, after the start of the toxin injections.

4.3. *Tissue processing*

One week after the last injection (week 8), mice were sacrificed by cervical dislocation, decapitated and trunk blood collected. The brains were removed and the rostral and caudal portions separated by an axial cut made across the whole brain at the tail end of the striatum. Both striata as well as a portion of cortex were removed and frozen at −80 °C until use. The remaining caudal brain portion was immediately fixed in 4% PFA for 3 days, placed in cryoprotectant and stored at −80 °C until use. The striatum was assayed for dopamine and urate by standard reverse phase high performance liquid chromatography with electrochemical detection as routinely performed in our laboratory (Chen et al., 2001; Ascherio et al., 2009). The striatum and cortex were used for purine and protein carbonyl assays, respectively. Fixed brains were cut on a Leica microtome into 30 μm-thick sections and stored for immunolabeling studies in a cryoprotectant consisting of 30% sucrose and 30% ethylene glycol in 0.1 M phosphate buffer. As previously described (Kachroo et al., 2010), sections were chromogenically stained for TH immunoreactivity (IR) followed by counterstaining with Nissl. Tyrosine hydroxylase-positive (TH+) and -negative (TH−) neurons were counted in the region of the SNpc.

4.4. *Striatal and serum sample preparation for HPLC purine analysis*

Brain samples were weighed and homogenized in 50 mM phosphoric acid, 0.1 mM EDTA, 50 μM methyl-DOPA (internal standard), and 1 μM DHBA (internal standard). Homogenates were centrifuged at 14,000 rpm for 15 min and the supernatant was transferred to a Costar SpinX (#8161) 0.22 μm CA filter tube and centrifuged at 14,000 rpm for 5 min. Samples were then stored at −80 °C until needed.

Whole blood was collected from the submandibular vein using Goldenrod animal lancets, and centrifuged at 14,000 rpm at 4 °C for 15 min. Serum was collected and de-proteinated by adding 0.4 M perchloric acid. The mixture was allowed to incubate on ice for 10 min and then centrifuged at 1400 rpm for 15 min. The supernatant was then collected and added to 0.2 M potassium phosphate. The resulting solution was added to a Costar SpinX (as above) and centrifuged at 14,000 rpm for 5 min. Samples were then stored at −80 °C until needed.

4.5. *Purine determination in striatal and serum samples*

A dual-pump gradient method was used to measure the concentrations of purines in the samples using a Varian Microsorb-MV reverse-phase column (150 × 4.6 mm, C_{18}, 5 μm pore size). Mobile phase A contained 0.52 mM sodium 1-pentanesulfonate and 0.20 M KH_2PO_4 monobasic at pH 3.5 using 85% phosphoric acid (HPLC-Grade, Fisher Scientific, Pittsburgh, PA). Mobile phase B had the same final concentrations as mobile phase A, except for the addition of 10% acetonitrile (v:v). All analyses were performed at a flow rate of 1 mL/min. The method ran at 0% B for 6 min and then linearly ramped to 70% B between 6 and 14 min. 70% B was maintained until 17.4 min, at which point, it returned to 0% B and was allowed to equilibrate until 20 min. The sample injection volume was 12 μL. Detection was performed by linking a UV–vis spectrophotometer upstream of two coulometric cells. The UV–vis detection was set to a wavelength of 254 nm. The first electrode was set to −100 mV and acted as conditioning cell. The analytical electrodes 1 and 2 were set at +150 and +450 mV, respectively. Data were collected using CoulArray Data Station 3.0 software (ESA Biosciences) with auto-range gain enabled. A standard curve was analyzed at the beginning of each run to determine the concentrations of purines in the biological samples. Methyl-DOPA and DHBA were used as internal standards to correct for minor variations between samples in the same run.

4.6. *Protein carbonyl assay*

Protein carbonyl levels were determined using the OxyBlot Protein Oxidation detection kit from Millipore. A 5 mg sample of cortex was removed from frozen brain tissue (kept frozen on dry ice) and added to a tube containing 40 μl of lysis buffer (RIPA buffer+50 mM DTT). The sample was hand homogenized, 5 μl of the resulting homogenate was added to 5 μl of SDS to a final concentration of 6% SDS. The samples were then derivatized by adding 10 μl of 1× 2,4-dinitrophenylhydrazine (DNPH) and incubated at room temperature for 15 min. The derivatization was halted using 7.5 μl of Neutralization buffer (aqueous solution of Trometamol and glycerol). The resulting solution was run on a 4% stacking/10% resolving polyacrylamide gel at 90 V until the samples had cleared the stacking gel, and then at 110 V for 1 h. The gel was then transferred to a nitrocellulose membrane. The membrane was then probed with a rabbit anti-

DNP (1:150) primary antibody and goat anti-rabbit IgG (1:300) secondary antibody for 1 h each. The blots were then visualized using horseradish peroxidase.

4.7. *Statistical analysis*

Optical density measurement for protein carbonyl level assessment was performed using the ImageJ software. All values are expressed as mean±SEM. For behavioral tests repeated measures ANOVA (RMANOVA) with *post hoc* analysis was performed. For all other analyses unpaired *t*-tests were performed.

Conflict of interest

No conflict of interest.

Acknowledgments

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PROTECTION BY INOSINE IN A CELLULAR MODEL OF PARKINSON'S DISEASE

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Abstract—Inosine (hypoxanthine 9-beta-D-ribofuranoside), a purine nucleoside with multiple intracellular roles, also serves as an extracellular modulatory signal. On neurons, it can produce anti-inflammatory and trophic effects that confer protection against toxic influences *in vivo* and *in vitro*. The protective effects of inosine treatment might also be mediated by its metabolite urate. Urate in fact possesses potent antioxidant properties and has been reported to be protective in preclinical Parkinson's disease (PD) studies and to be an inverse risk factor for both the development and progression of PD. In this study we assessed whether inosine might protect rodent MES 23.5 dopaminergic cell line from oxidative stress in a cellular model of PD, and whether its effects could be attributed to urate. MES 23.5 cells cultured alone or in presence of enriched murine astroglial cultures MES 23.5–astrocytes co-cultures were pretreated with inosine (0.1–100 μM) for 24 h before addition of the oxidative stress inducer H_2O_2 (200 μM). Twenty-four hours later, cell viability was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or immunocytochemistry in pure and MES 23.5–astrocytes co-cultures, respectively. H_2O_2-toxic effect on dopaminergic cells was reduced when they were cultured with astrocytes, but not when they were cultured alone. Moreover, in MES 23.5–astrocytes co-cultures, indicators of free radical generation and oxidative damage, evaluated by nitrite (NO_2^-) release and protein carbonyl content, respectively, were attenuated. Conditioned medium experiments indicated that the protective effect of inosine relies on the release of a protective factor from inosine-stimulated astrocytes. Purine levels were measured in the cellular extract and conditioned medium using high-performance liquid chromatography (HPLC) method. Urate concentration was not significantly increased by inosine treatment however there was a significant increase in levels of other purine metabolites, such as adenosine, hypoxanthine and xanthine. In particular, in MES 23.5–astrocytes co-cultures, inosine medium content was reduced by 99% and hypoxanthine increased by 127-fold. Taken together these data raise the possibility that inosine might have a protective effect in PD that is independent of any effects mediated through its metabolite urate.

Key words: MES 23.5 cells, astrocytes, urate, HPLC, cell viability, oxidative stress.

INTRODUCTION

Inosine is a purine shown to have trophic protective effects on neurons and astrocytes subjected to hypoxia or glucose-oxygen deprivation (Haun et al., 1996) and to induce axonal growth following neuronal insult *in vivo* and *in vitro* (Zurn and Do, 1988; Benowitz et al., 1998; Petrausch et al., 2000; Chen et al., 2002; Wu et al., 2003). Moreover, inosine showed anti-inflammatory effects in the central nervous system (CNS) and periphery (Jin et al., 1997; Hasko et al., 2000; Gomez and Sitkovsky, 2003; Shen et al., 2005; Rahimian et al., 2010). Some (Toncev, 2006; Markowitz et al., 2009) but not all (Gonsette et al., 2010) clinical studies have suggested a possible antioxidant protective effect of inosine in multiple sclerosis patients (Markowitz et al., 2009). In these trials inosine consistently elevated serum urate, which was proposed to mediate any protective effect of inosine (Markowitz et al., 2009; Spitsin et al., 2010).

Oxidative stress is thought to be a key pathophysiological mechanism in Parkinson's disease (PD) leading to cellular impairment and death (Ross and Smith, 2007). Urate – a major antioxidant circulating in the human body – has emerged as an inverse risk factor for PD. Clinical and population studies have found the urate level in serum or CSF to correlate with a reduced risk of developing PD in healthy individuals and with a reduced risk of clinical progression among PD patients (Weisskopf et al., 2007; Schwarzschild et al., 2008; Ascherio et al., 2009). Moreover, in cellular and animal models of PD, urate elevation has been shown to reduce oxidative stress and toxicant-induced loss of dopaminergic neurons (Wang et al., 2010; Cipriani et al., 2012a,b; Gong et al., 2012; Zhu et al., 2012; Chen et al., 2013). Although inosine can elevate urate concentration in the periphery in animals and humans, little is known about its effect on the urate level in the CNS (Ceballos et al., 1994; Scott et al., 2002; Rahimian et al., 2010; Spitsin et al., 2010). A cellular study indicated that inosine added to cortical astroglial (but not neuronal) cultures increases urate concentration in the medium (Ceballos et al., 1994).

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Abbreviations: CNS, central nervous system; DHBA, 3,4-dihydroxybenzylamine; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PD, Parkinson's disease; SDS, sodium dodecyl sulfate.

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In the present study we characterized a protective effect of inosine on oxidative stress-induced dopaminergic cell death in a cellular model of PD and investigated whether urate elevation might mediate the effect.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6 mice were employed to obtain astroglial cultures. All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals with approval from the animal subjects review board of the Massachusetts General Hospital.

MES 23.5 cell line

The rodent MES 23.5 dopaminergic cell line (Crawford et al., 1992) was obtained from Dr. Weidong Le at the Baylor College of Medicine (Houston, USA). MES 23.5 cells were cultured on polyornithine-coated T75 flasks (Corning Co, Corning, NY) in culture medium; Dulbecco modified Eagle medium (DMEM, Invitrogen/Gibco), added with Sato components (Sigma Immunochemicals), and supplemented with 2% newborn calf serum (Invitrogen), 1% fibroblast growth factor (Invitrogen), penicillin 100 U ml^{-1} and streptomycin 100 μg mL^{-1} (Sigma), at 37 °C in a 95% air–5% carbon dioxide, humidified incubator. Culture medium was changed every 2 days. At confluence, MES 23.5 cells were either sub-cultured new T-75 flasks or used for experiments. For experiments, MES 23.5 cells were seeded at a density of 600 cells per mm^2 onto polyornithine-coated plates or flasks (according to the assay, see below) in culture medium. Twenty-four hours later, it was changed to DMEM serum-free medium. At this time, increasing concentrations of inosine (0–100 μM) were added to the cultures for 24 h and again during toxicant treatment. 200 μM H_2O_2 wasadded to the cultures for 24 h and then cells were used for assays.

Enriched astroglial cultures

Astroglial cultures were prepared from the brains of 1- or 2-day-old neonatal mice as previously described (Cipriani et al., 2012b). Briefly, cerebral cortices were digested with 0.25% trypsin for 15 min at 37° C. The suspension was pelleted and re-suspended in culture medium (DMEM, fetal bovine serum (FBS) 10%, penicillin 100 U ml^{-1} and streptomycin 100 μg ml^{-1} to which 0.02% deoxyribonuclease I was added). Cells were plated at a density of 1800 cells per mm^2 on poly-L-lysine (100 μg ml^{-1})/DMEM/F12-coated flasks and cultured at 37 °C in humidified 5% CO_2 and 95% air for 7–10 days until reaching confluence.

In order to remove non-astroglial cells, flasks were agitated at 200 rpm for 20 min in an orbital shaker and treated with 10 μM cytosine arabinoside (Ara-C) dissolved in cultured medium for 3 days. After the treatment, astrocytes were subjected to mild trypsinization (0.1% for 1 min) and then sub-plated (120 cells per mm^2) onto poly-L-lysine (100 μg ml^{-1})/DMEM/F12-coated plates or flasks (according to the assay, see below) in DMEM plus 10% FBS for assays. Astroglial cultures comprised >95% astrocytes, <2% microglial cells and <1% oligodendrocytes; no neuronal cells were detected (Cipriani et al., 2012b).

MES 23.5–astrocytes co-cultures

MES 23.5 cells were cultured on a layer of enriched astroglial cultures prepared as described above. Briefly, astrocytes were allowed to grow for 48 h on poly-L-lysine (100 μg ml^{-1})/DMEM/F12-coated plates or flasks (according to the assay, see below) in DMEM plus 10% FBS. Then, MES 23.5 cells were seeded on top at a concentration of 600 cells per mm^2 in MES 23.5 culture medium. An astrocyte:MES 23.5 cell ratio of 1:5 was chosen on the basis of our previous observations (Cipriani et al., 2012b), which indicated this proportion of astrocytes as sufficiently low to avoid a direct effect of astrocytes on dopaminergic cell survival. Twenty-four hours later, medium was changed to DMEM serum-free medium and subjected to treatments. Inosine was added to the cultures 24 h before and during 200 μM H_2O_2 treatment. In our previous study this H_2O_2 concentration was shown to have no effect on astrocyte viability (Cipriani et al., 2012b). At the end of treatment, MES 23.5 cells were easily detached from astrocytes and dissociated by gently pipetting up and down the medium before processing for biochemical assays.

Conditioned media experiments

Enriched-astrocyte cultures were grown on poly-L-lysine (100 μg ml^{-1})/DMEM/F12-coated 6 well-plates in DMEM plus 10% FBS. Astrocytes were allowed to grow for three days and then the medium was changed to MES 23.5 culture medium in order to reproduce co-culture conditions. The day after, medium was changed to DMEM containing 100 μM inosine or vehicle. Twenty-four hours later, conditioned medium was collected and filtered through a 0.2 μM membrane to remove cellular debris. MES 23.5 cells were treated with increasing concentrations of conditioned medium 24 h before and during H_2O_2 treatment.

Drugs

Inosine was dissolved in DMEM as 20× concentrated stocks. H_2O_2 was dissolved in PBS (0.1 M, pH 7.4) as 100× concentrated stocks. Drugs were obtained from Sigma.

Cell viability and toxicity assessments

In MES 23.5 cultures, cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma). This assay is based on the conversion of the yellow tetrazolium salt MTT by mitochondrial dehydrogenase of live cells to the purple formazan (Hansen et al., 1989). Briefly, MES 23.5 cells were cultured in polyornithine-coated 96-well plates (600 cells per mm^2) and grown for at least 24 h. Then, the medium was changed to DMEM serum-free medium

for 24 h before H_2O_2 was added. In order to assess inosine protection, increasing concentrations of drug (0–100 μM) were loaded 24 h before and again during toxicant treatment. After washes, 100 μl of MTT solution (0.5 mg ml^{-1} in DMEM) was added for 3 h at 37 °C. Then, MES 23.5 cells were lysed with 10 μl/well of acidic isopropanol (0.01 M HCl in absolute isopropanol) to extract formazan that was measured spectrophotometrically at 490 nm with a Labsystems iEMS Analyzer microplate reader.

In MES 23.5–astrocytes co-cultures, surviving MES 23.5 cells were quantified by immunocytochemistry (Lotharius et al., 2005; Dumitriu et al., 2011; Cipriani et al., 2012b). MES 23.5 were grown on top of astrocytes in 96-well plates as described above. Increasing concentrations of drug (0–100 μM) were loaded 24 h before and again during toxicant treatment. After washing in PBS, cultures were fixed with 4% (wt/vol) paraformaldehyde for 1 h at room temperature. Then, cells were incubated with an Alexa 488-conjugated antibody specific for neuronal cells, Milli-Mark FluoroPan Neuronal Marker, (Millipore; 1:200, overnight at 4 °C). Fluorescence was read at 535 nm by using a microplate reader.

High-performance liquid chromatography (HPLC)

To determine purine content in cells and medium samples, MES 23.5, MES 23.5–astrocytes co-cultures or enriched astroglial cultures were prepared as described above and cultured in T75 flasks. Purine content was determined using our previously described HPLC-based analytical methods (Burdett et al., 2013). Briefly, cell medium was collected and added with 30% vol/vol of a buffer containing 150 mM phosphoric acid, 0.2 mM EDTA, and 1 μM 3,4-dihydroxybenzylamine (DHBA; used as internal standard). Cells were collected after washing in ice-cold PBS and purines were extracted in the same buffer used for medium. Samples were then filtered through a 0.2-μm Nylon microcentrifuge filter (Spin-X, Corning) at 4 °C. Samples were maintained at 4 °C and injected using an ESA Biosciences (Chelmsford, MA) autosampler, and chromatographed by a multichannel electrochemical/UV HPLC system with effluent from the above column passing through a UV–VIS detector (ESA model 528) set at 254 nm and then over a series of electrodes set at −100 mV, +250 mV and +450 mV. To generate a gradient two mobile phases were used. Mobile phase A consisted of 0.2 M potassium phosphate and 0.5 mM sodium 1-pentanesulfonate; mobile phase B consisted of the same plus 10% (vol/vol) acetonitrile. Mobile phase B increased linearly from 0% to 70% between 6th and 14th min of the run.

Nitrite (NO_2^-) release

MES 23.5–astrocytes co-cultures were grown on a 96-well plate as described above. After treatments, nitrite release (NO_2^-), an indicator of free radical generation, was quantified in cell medium by the Griess assay. An azo dye is produced in the presence of nitrite by the Griess reaction and colorimetrically detected. Briefly, 100 μl of supernatant collected from treated cultures were added to 100 μl of Griess reagent (Sigma) and absorbance was read at 540 nm with a microplate reader. Blanks were prepared by adding medium containing toxicants and/or protectants to the Griess solution.

Protein carbonyl protein assay

MES 23.5–astrocytes co-cultures were grown in 6-well plates as described above. After treatments, cultures were washed with ice-cold PBS and oxidized proteins were detected in MES 23.5 cells, using the Oxyblot assay kit (Chemicon). MES 23.5 cells were detached from astrocytes in ice-cold PBS, spun to form a pellet at 4 °C and resuspended in ice-cold RIPA buffer containing 50 mM DTT. Cells were allowed to lyse on ice for 15′. For the assay, 20 μg of protein were derivatized in 10 μL of 2,4-dinitrophenylhydrazine (DNPH). After derivatization samples were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel (10% [wt/vol] acrylamide, 0.1% [wt/vol] SDS) and transferred electrophoretically onto 0.2 μ nitrocellulose membranes. Membranes were loaded with an antibody specific to dinitrophenylhydrazone moiety of the proteins and reaction visualized by chemiluminescence.

Protein detection

After treatment, cells were washed in ice-cold PBS, collected and resuspended in ice-cold Ripa buffer. Cells were incubated on ice for 15', followed by sonication for complete lysis. Proteins were quantified in 4 μl of each sample using Bio-Rad Protein Assay reagent (Biorad Laboratories) and measured at 600 nm with a microplate reader.

Statistical analysis

Statistical analysis was performed by GraphPad Prism version 4.00 (GraphPad Software Inc.). Unpaired Student's *t*-test was used when two group samples were compared. ANOVA analysis followed by Newman–Keuls was used when more than two group samples were compared. Values were expressed as mean ± SEM. Differences with a $P < 0.05$ were considered significant and indicated in figures by symbols explained in legends.

RESULTS

Astrocytes mediated protective effect of inosine on dopaminergic cells

Previously we showed that urate protected a dopaminergic cell line (MES 23.5) against oxidative stress when cells were cultured with astrocytes (Cipriani et al., 2012b). To assess whether inosine protected the dopaminergic cell line in a similar way we tested inosine on MES 23.5 cells cultured alone or with cortical astrocytes (MES 23.5–astrocytes co-cultures) treated with 200 μM H_2O_2.

Inosine on its own had no effect on MES 23.5 viability (one-way ANOVA, $P > 0.05$) (Fig. 1A), and showed only a trend toward modest protection with increasing

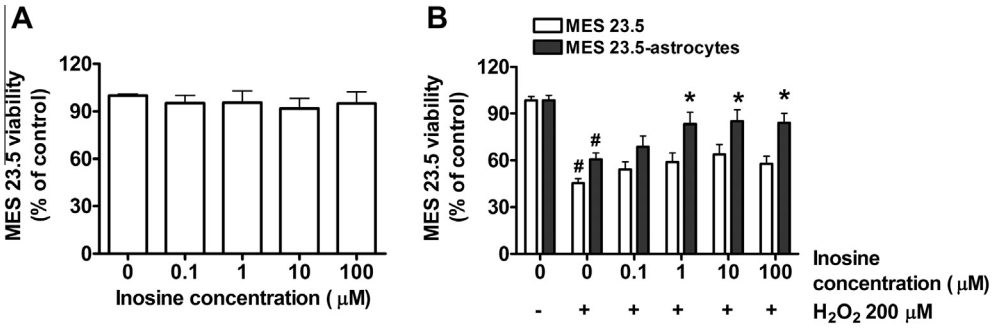

Fig. 1. Astrocytes potentiated the protective effect of inosine on 200 μM H_2O_2-treated MES 23.5 cells. (A) Viability of MES 23.5 cells treated for 24 h with increasing concentrations of inosine (0–100 μM). (B) Effect of inosine treatment (0–100 μM) at 24 h of toxic treatment with 200 μM H_2O_2 on viability of MES 23.5 cells cultured alone (white bars) or in the presence of astrocytes (gray bars). Cultures were treated with inosine 24 h before and during toxic treatment. Data represent mean ± SEM of values from four experiments, each of which yielded a mean of triplicate determinations for each condition. One-way ANOVA analysis ($P < 0.001$) followed by Newman–Keuls multiple comparison test ($^{\#}P < 0.01$ vs respective '0 inosine/ $-H_2O_2$' value; $^{*}P < 0.05$ vs '0 inosine/ $+H_2O_2$' value).

concentrations from 0.1 to 100 μM against H_2O_2 toxicity (one-way ANOVA, $P > 0.05$) in pure MES 23.5 cultures. However, in the presence of a relatively low density of astrocytes (plated at a density of 120 cells per mm^2), MES 23.5 cell viability significantly increased in comparison to inosine-untreated cells ($P < 0.05$; Fig. 1B).

Inosine decreased toxicant-induced oxidative stress

To determine whether protection was associated with reduced oxidative stress and protein damage, we measured the effect of inosine on oxidative stress markers in H_2O_2-treated co-cultures of MES 23.5 cells and astrocytes. At 24 h, inosine decreased the level of NO_2^- (nitrite), an indicator of free radical generation, from 2-fold to 1.4-fold of the control value in cell medium ($P = 0.00139$, Fig. 2A). Moreover, at 3 h inosine decreased protein oxidation, measured as protein carbonyl content in MES 23.5 cells (after removal from astrocytes), from 4.6- to 2.7-fold of control value ($P = 0.002$) (Fig. 2B).

Protection mediated by astrocytes does not require their physical contact with dopaminergic cells

We previously observed that astrocytes mediate urate's protective effect through the release of protective factor(s). To assess if astrocytes mediated inosine's protective effect in the same fashion, MES 23.5 cells were treated with increasing percentages of medium collected from untreated or inosine-treated astrocytes. Medium from untreated astrocytes did not show a statistically significant effect on H_2O_2-treated MES 23.5 viability at any given concentration ($P > 0.05$). On the other hand, conditioned medium from astrocytes treated for 24 h with 100 μM inosine improved MES 23.5 viability in a concentration-dependent manner ($P < 0.001$). This observation was confirmed by a two-way ANOVA analysis that showed significant effect of conditioned medium ($F_{1,151} = 46.28$, $P < 0.0001$) and conditioned medium percentage ($F_{1,151} = 7.31$, $P < 0.0001$) and significant interaction between these two factors ($F_{1,151} = 3.59$, $P = 0.0079$; Fig. 3).

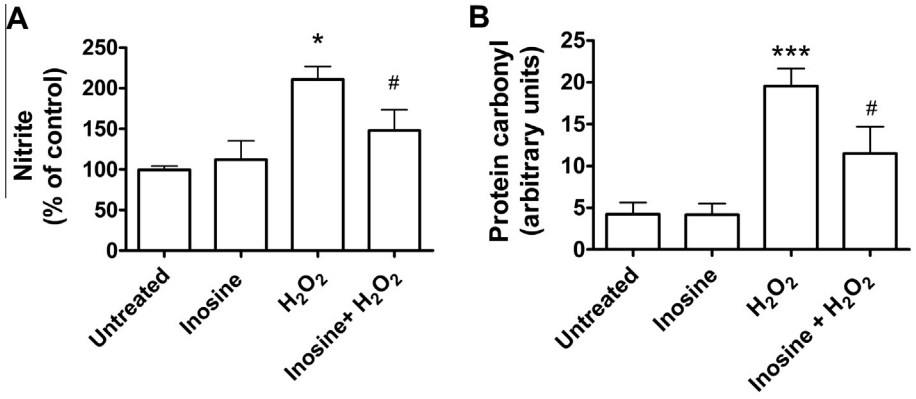

Fig. 2. Inosine reduced oxidative stress in MES 23.5 cells cultured with astrocytes. (A) Effect of inosine treatment (0–100 μM) on 200 μM H_2O_2 induced NO_2^- release in the medium of MES 23.5–astrocytes co-cultures at 24 h of toxic treatment. Cultures were treated with inosine 24 h before and during toxic treatment. Data represent mean ± SEM of three triplicate experiments. (B) Effect of inosine treatment (0–100 μM) on 200 μM H_2O_2 induced protein carbonylation in MES 23.5 cells cultured with astrocytes at 3 h of toxic treatment. Cultures were treated with inosine 24 h before and during toxic treatment. Data represent mean ± SEM of six replicates over three independent experiments. One-way ANOVA: $^{*}P < 0.05$ and $^{***}P < 0.001$ vs untreated and inosine (alone) values; $^{\#}P < 0.05$ vs H_2O_2 (alone) value.

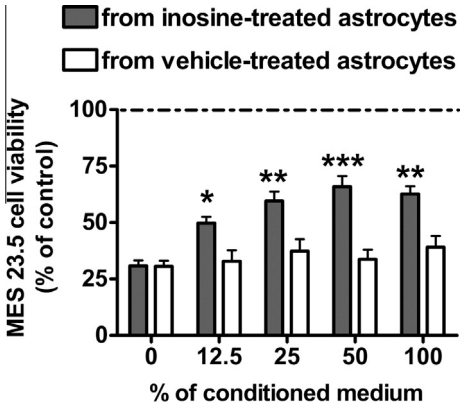

Fig. 3. Inosine-conditioned medium from astrocytes increased viability of H_2O_2-treated MES 23.5 cells. Effect of increasing concentration of cell medium collected from control (white bars) or 100 μM of inosine-treated astrocytes (gray bars) on 200 μM H_2O_2-induced cell death in MES 23.5 pure cultures. Cultures were treated with conditioned medium 24 h before and during toxic treatment. Data represent mean ± SEM of thirteen independent experiments. Two-way ANOVA analysis ($P = 0.0003$) followed by the Newman–Keuls multiple comparison test ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs respective control value).

Inosine treatment did not affect urate concentration

Extracellular inosine breakdown has been reported in astroglial cultures (Ceballos et al., 1994). To determine whether inosine degradation occurred in our cultures, purine metabolites of inosine were measured in the medium of MES 23.5–astroglial co-cultures treated with inosine. For this experiment we selected two time points: 0, when inosine was added to the cultures, and 24 h, when the cultures would be treated with toxicant. Over 24 h inosine concentration, reflecting both endogenous plus exogenous contributions, was reduced by 99% ($P < 0.0001$); over the same time period hypoxanthine and xanthine increased by 127-fold ($P < 0.0001$) and 1.5-fold ($P < 0.0001$), in comparison to time zero, respectively (Table 1). Thus, the hypoxanthine increment was 1.6-fold greater than the amount of inosine added.

Moreover, adenosine, an inosine 'precursor', increased by 4-fold ($P = 0.0001$, Table 1) over the 24 h. By contrast, urate content was not changed in the medium over the same time period ($P = 0.46$, Table 1), indicating that extracellular urate unlikely mediated inosine's effects.

In our previous studies we found evidence that urate's protective effect on dopaminergic cells was correlated with its increase within astrocytes (Cipriani et al., 2012b). To assess whether inosine treatment increased intracellular urate in astroglial cells its concentration was measured in inosine-treated enriched astroglial cultures at time 0 and 24 h of treatment. Although adenosine increased 2-fold, intracellular concentrations of urate and other purines were not changed at 24 h in comparison to time 0 (Table 2) and vehicle-treated cells (data not shown). Similarly, no effect was seen on extracellular urate, where inosine induced an approximately 5-fold increase in hypoxanthine concentration ($P < 0.01$, Table 3). Thus despite the expression of functional xanthine oxidase, the enzyme that converts hypoxanthine to xanthine and in turn to urate in cortical astrocytes (Ceballos et al., 1994), we did not find evidence of the conversion of inosine to urate.

Purine increase induced by inosine in mixed-cultures might play a role in inosine protective effect. To assess whether this effect was selective for mixed-cultures, inosine metabolite concentration was also measured in the medium of MES 23.5 cultures after inosine treatment. Similarly to mixed cultures, in MES 23.5 cultures inosine concentration decreased to about 30% ($P < 0.0001$) and 3% ($P < 0.0001$) of control at 6 and 24 h, respectively. Hypoxanthine increased over the time up to 4-fold ($P < 0.0001$) at 24 h in comparison to time zero and xanthine by 1.8-fold in comparison to 6 h (Table 4). Moreover, adenosine increased about 9-fold ($P < 0.0001$) in comparison to time zero. Urate concentration did not change at any tested time (Table 4). These data exclude a direct effect of inosine metabolites on MES 23.5 cells since no protective effect was found in these experimental conditions.

DISCUSSION

We report that inosine prevented oxidative stress-induced cell death in dopaminergic MES 23.5 cells cultured with astrocytes. This effect appeared to be independent of increased intracellular urate, an inosine metabolite and established antioxidant.

Table 1. Extracellular purine content at time zero (0) and 24 h in 100 μM inosine-treated MES 23.5–astrocytes co-cultures

Analites	Concentration (μM)	
	0	24 h
Adenosine	0.16 ± 0.01	0.82 ± 0.04***
Inosine	104 ± 8	0.74 ± 0.09***
Hypoxanthine	1.25 ± 0.26	160 ± 28***
Xanthine	0.34 ± 0.03	0.85 ± 0.05***
Urate	0.89 ± 0.04	0.83 ± 0.05

Purine content of co-cultures cell medium was analyzed by high-performance liquid chromatography. Data are expressed as μM. Significance was determined by Student's *t* test: $^{***}P < 0.001$ vs 0 time point value. Data are presented as mean ± SEM of eight experiments.

Table 2. Intracellular purine content at time zero (0) and 24 h in 100 μM inosine-treated enriched astroglial cultures

Analites	Concentration (nmol/g of protein)	
	0	24 h
Adenosine	130 ± 48	390 ± 137*
Inosine	607 ± 230	477 ± 204
Hypoxanthine	320 ± 77	400 ± 199
Xanthine	3 ± 1	6 ± 3
Urate	38 ± 5	26 ± 2

Purines were extracted from enriched astroglial cultures by cell trituration in extracting buffer (see methods) and measured by high-performance liquid chromatography. Data are expressed as nmol/g of protein. Significance was determined by Student's *t* test: $^{*}P = 0.012$. Data are presented as mean ± SEM of four experiments.

Table 3. Extracellular purine content at time zero (0) and 24 h in 100 μM inosine-treated enriched astroglial cultures

Analites	Concentration (μM)	
	0	24 h
Adenosine	1.7 ± 0.1	1.6 ± 0.4
Inosine	105 ± 1	9.0 ± 0.1***
Hypoxanthine	22.6 ± 0.1	127 ± 21**
Xanthine	8.5 ± 0.1	13 ± 2
Urate	20.3 ± 0.1	24 ± 4

Purine content of astrocyte medium was measured by high-performance liquid chromatography. Data are expressed as μM. Significance was determined by Student's *t* test: $^{**}P < 0.01$ and $^{***}P < 0.001$ vs 0 time point value. Data are presented as mean ± SEM of four experiments.

Table 4. Extracellular purine content in 100 μM inosine-treated MES 23.5 cells over 24 h of treatment

Analites	Concentration (μM)		
	0	6 h	24 h
Adenosine	0.28 ± 0.09	2.28 ± 0.08	2.8 ± 0.4**
Inosine	89 ± 20	30 ± 2**	2.6 ± 0.1***
Hypoxanthine	149 ± 10	680 ± 43***	780 ± 81***
Xanthine	N.D.	0.10 ± 0.04	0.28 ± 0.07*
Urate	N.D.	N.D.	N.D.

Purine content of astrocyte medium was measured by high-performance liquid chromatography.
Data are expressed as μM. Student's *t* test, $n = 8$, $^{*}P < 0.05$ vs 6 h value. One-way ANOVA followed by Newman–Keuls test: $^{**}P < 0.01$ and $^{***}P < 0.001$ vs 0 time point value. Data are presented as mean ± SEM of eight experiments.

As a close structural homolog of adenosine, inosine may confer protection by direct mechanisms, activating multiple subtypes of adenosine receptors that are known to modulate cell death. Several studies have implicated A_1, A_{2A} or A_3 receptors as mediators of inosine effects in the setting of inflammatory or ischemic injury (Jin et al., 1997; Gomez and Sitkovsky, 2003; Shen et al., 2005; Rahimian et al., 2010). For example, inosine was found to reduce ischemic brain injury in rats likely via an adenosine A_3 receptor-dependent pathway (Shen et al., 2005).

In vitro studies showed inosine to be protective in models of hypoxia (Litsky et al., 1999) and glucose–oxygen deprivation (Haun et al., 1996) where it mediated adenosine protective effects. Inosine has been shown to protect neurons with a neurotrophic effect, promoting axonal regeneration *in vivo* and *in vitro* (Zurn and Do, 1988; Chen et al., 2002; Wu et al., 2003) and inducing the expression of axonal growth-associated genes (Benowitz et al., 1998; Petrausch et al., 2000). This neuroprotective effect can be exerted with a receptor-independent mechanism, for example, activating the cytoplasmic protein kinase Mst3b as shown in the setting of stroke or traumatic brain injury in rodents (Zai et al., 2011). *In vitro* and *in vivo* studies showed inosine to have anti-inflammatory effects in inflammatory or ischemic injury (Jin et al., 1997; Hasko et al., 2000; Gomez and Sitkovsky, 2003; Shen et al., 2005; Rahimian et al., 2010). Moreover, a clinical study raised the possibility that inosine may have antioxidant properties improving structural and neurological impairment in multiple sclerosis patients (Markowitz et al., 2009).

A previous study reported that inosine protection against chemical hypoxia was dependent on the presence of astrocytes in cultures (Litsky et al., 1999). Similarly, we show that inosine's protective effect on dopaminergic cells was mediated by astrocytes, suggesting a mechanism more complex than a direct protective effect exerted by inosine. Moreover, the rapid inosine degradation occurring in cultures would suggest more of an indirect effect of inosine, which would be consistent with stimulated production and release of an astrocytic protective factor(s) (Imamura et al., 2008).

The rapid elimination of exogenous inosine and increase in its precursor and metabolites are also consistent with the possibility that a purine related to inosine mediates its protective effect. Treatment with inosine at a high concentration relative to endogenous levels increased the concentration of its precursor adenosine in co-cultures, suggesting either conversion of inosine into adenosine (Murray, 1971) or feedback inhibition of adenosine deaminase (Meyskens and Williams, 1971) leading to reduced degradation of endogenous adenosine. Extracellular adenosine in turn may act on its own receptors to enhance survival of dopaminergic neurons in cultures (Michel et al., 1999) or it can be taken up by neurons (Hertz and Matz, 1989).

Alternatively, increased metabolism of inosine may have mediated its protective effect. Inosine breakdown protected cells subjected to glucose deprivation or hypoxia-reoxygenation preserving cellular ATP content (Jurkowitz et al., 1998; Shin et al., 2002; Módis et al., 2009; Szoleczky et al., 2012). Intracellular inosine (and adenosine by way of inosine) was shown to be transformed to hypoxanthine and ribose 1-phosphate by purine nucleotides phosphorylase (Jurkowitz et al., 1998). In turn, ribose 1-phosphate was converted to an intermediate that can enter the anaerobic glycolytic pathway providing the ATP necessary to maintain cell integrity (Jurkowitz et al., 1998). Inhibition of the enzyme purine nucleoside phosphorylase notably prevented the neuroprotective effect of inosine in glial cells and mixed astrocyte-neuronal cultures (Jurkowitz et al., 1998; Litsky et al., 1999). Moreover, this pathway can represent the primary energy source for erythrocytes lacking functional glucose transporters (Young et al., 1985). In our study we found that the hypoxanthine increment was about 24-times higher in MES 23.5-astrocytes co-cultures than in MES 23.5 cells alone after inosine treatment. Purine nucleoside phosphorylase is highly expressed in astrocytes (Ceballos et al., 1994); thus the presence of astrocytes in cultures might provide conditions sufficient for enhanced ATP production during the toxic insult. This raises the possibility that the anaerobic glycolytic pathway might contribute to the protective effect of inosine on dopaminergic cells during oxidative stress. A role for this pathway and the associated production of hypoxanthine by increased purine nucleoside phosphorylase activity in astrocytes may also account for the observed hypoxanthine increase in molar excess of

exogenous inosine introduced. Regardless of whether altered cellular energy metabolism induced by inosine breakdown or a specific metabolite of inosine is protective, these scenarios support the hypothesis that inosine treatment might induce release of factor(s) from astrocytes to protect dopaminergic cells.

Inosine has been shown to be converted to urate in cultures (Ceballos et al., 1994) and to elevate urate serum level in rodents and humans (Ceballos et al., 1994; Scott et al., 2002; Rahimian et al., 2010; Spitsin et al., 2010). Although we observed higher extracellular concentrations of inosine's metabolites, such as hypoxanthine, we did not find increased urate levels in media or in astrocytes. It is unlikely that an earlier increase in urate was missed due to its being metabolized to allantoin since we have already shown that cortical astrocytes and MES 23.5 cells do not express urate oxidase, the enzyme that converts urate to allantoin (Cipriani et al., 2012b). Together these observations argue against a role for urate as the mediator of inosine's protective effects in this cellular model of oxidative stress in PD. However, purine metabolism is of course different in intact humans versus murine culture models and the present findings of a urate-independent protective effect in culture do not preclude the protective effect of urate, which can be substantially elevated in people treated with inosine (The Parkinson Study Group SURE-PD Investigators et al., 2014).

In PD the degeneration of dopaminergic neurons is thought to be induced by accumulation of oxidative damage that leads to mitochondrial impairment and protein aggregation. The finding that inosine prevents oxidant-induced dopaminergic cell loss may be of substantial epidemiological and therapeutic significance for PD. A phase II clinical trial of inosine in early PD showed that inosine was safe, tolerable and effective in raising CSF and serum urate levels (The Parkinson Study Group SURE-PD Investigators et al., 2014). Our results suggest that if CNS inosine itself was elevated in the CNS of treated individuals it could produce a neuroprotective effect independent of urate.

CONCLUSIONS

Inosine had antioxidant and protective effects on dopaminergic cells with a mechanism that does not require increased urate concentration. This finding further supports inosine as a candidate for PD therapy.

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Appendix B

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Chronic Treatment with Novel Small Molecule Hsp90 Inhibitors Rescues Striatal Dopamine Levels but Not α-Synuclein-Induced Neuronal Cell Loss

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Abstract

Hsp90 inhibitors such as geldanamycin potently induce Hsp70 and reduce cytotoxicity due to α-synuclein expression, although their use has been limited due to toxicity, brain permeability, and drug design. We recently described the effects of a novel class of potent, small molecule Hsp90 inhibitors in cells overexpressing α-synuclein. Screening yielded several candidate compounds that significantly reduced α-synuclein oligomer formation and cytotoxicity associated with Hsp70 induction. In this study we examined whether chronic treatment with candidate Hsp90 inhibitors could protect against α-synuclein toxicity in a rat model of parkinsonism. Rats were injected unilaterally in the substantia nigra with AAV8 expressing human α-synuclein and then treated with drug for approximately 8 weeks by oral gavage. Chronic treatment with SNX-0723 or the more potent, SNX-9114 failed to reduce dopaminergic toxicity in the substantia nigra compared to vehicle. However, SNX-9114 significantly increased striatal dopamine content suggesting a positive neuromodulatory effect on striatal terminals. Treatment was generally well tolerated, but higher dose SNX-0723 (6–10 mg/kg) resulted in systemic toxicity, weight loss, and early death. Although still limited by potential toxicity, Hsp90 inhibitors tested herein demonstrate oral efficacy and possible beneficial effects on dopamine production in a vertebrate model of parkinsonism that warrant further study.

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Introduction

Protein aggregates such as beta amyloid in Alzheimer's disease, tau deposits in frontotemporal dementia, and Lewy bodies in Parkinson disease (PD) are a common pathological feature in neurodegenerative disorders. Molecular chaperones, such as heat shock proteins, co-localize with aggregates in neurodegenerative disease and play a critical role in protein processing and homeostasis [1,2]. Heat shock proteins (Hsp) such as Hsp70 direct misfolded and potentially toxic proteins for degradation via the proteasome or autophagy-lysosomal system [3–5]. Furthermore, induction of Hsp70 is protective in models of neurodegenerative disorders, such as Huntington's disease, spinocerebellar ataxias, and tauopathy disorders (i.e., Alzheimer's disease) [6–8]. We and others have demonstrated that Hsp70 can enhance the degradation of misfolded α-synuclein, reduce oligomer formation, and mediate toxicity due to α-synuclein overexpression [9–11]. Moreover, direct pharmacological upregulation of Hsp70 with geldanamycin, an Hsp90 inhibitor, results in decreased cytotoxicity from α-synuclein [12]. Thus targeting molecular chaperones, such as Hsp70 or Hsp90, has reasonable therapeutic potential not only for parkinsonism, but also for related neurodegenerative disorders.

A number of small molecule inhibitors of Hsp90 have been tested in models of PD and other neurodegenerative disorders [13,14]. Hsp90 negatively regulates Hsp70 expression by blocking

activation of the transcription factor HSF-1; thus inhibitors result in Hsp70 induction [15]. Geldanamycin is a naturally occurring benzoquinone that blocks Hsp90 interaction with HSF-1 resulting in enhanced Hsp70 expression [16]. However, its utility is limited by hepatotoxicity and poor brain permeability. In contrast, the analogues 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG) have greater potency, reduced toxicity, and cross the blood brain barrier more efficiently [6,17]. Preliminary testing also showed neuroprotection in models of polyglutamine disorders. However, despite promising effects in clinical trials for cancer, these compounds have been pursued only in a limited fashion due to hepatotoxicity, poor oral bioavailability, and formulation issues [18,19].

Recently, a novel class of Hsp90 inhibitors with structure different from that of geldanamycin and derivatives was discovered among a screen for drugs that bind the ATP pocket of Hsp90. SNX-2112 (4-[6,6-dimethyl-4-oxo-3-(trifluoromethyl)-4,5,6,7-tetrahydro-1H-indazol-1-yl]-2-[(trans-4-hydroxycyclohexyl)amino]-benzamide; PF-04928473) was the initial drug described and exhibited potent Hsp90 inhibition, anti-tumor activity, blood-brain permeability, and oral bioavailability [20,21]. We recently tested compounds from the same class in a PD cell model [22]. Several of these novel Hsp90 inhibitors, in particular SNX-0723 (PF-04924868), significantly reduced α-synuclein oligomer formation and cytotoxicity concomitant with Hsp70 induction. SNX-0723 also exhibited favorable pharmacokinetic properties and induced Hsp70 in rat brain [22]. Based on these findings we next wanted to test the effect of these novel Hsp90 inhibitors in a rat model of parkinsonism. We and others have demonstrated that AAV expression–utilizing a variety of viral serotypes: 1, 2, 5, 6, and 8–of α-synuclein results in progressive, dopaminergic nigrostriatal neurodegeneration over the course of several weeks [23–25]. This model allowed us to test whether chronic oral administration of novel Hsp90 inhibitors in rats could protect against progressive α-synuclein-induced nigrostriatal toxicity.

Methods

Viral Production

Construction of rAAV vectors used to express human wild-type α-synuclein was as previously described (AAV-CBA-Syn-WPRE construct) [26]. Recombinant AAV2/8 virus was generated by the Harvard Gene core (Harvard Gene Therapy Initiative, Harvard Medical School) via tripartite transfection of the *cis*-transgene, packaging (*rep* and *cap*) genes, and helper plasmid into HEK 293A cells. Viral particles were purified by iodixanol density gradient, isolated, and titered by dot blot hybridization. Final titer for rAAV expressing human α-synuclein was 5.6×10^{12} gc/mL.

Stereotaxic Surgery and Drug Treatment

Animal protocols and procedures were approved by the MGH Subcommittee on Research Animal Care (IACUC #2005N000156) and followed recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. All surgery was performed under ketamine/xylazine anesthesia, and all efforts were made to minimize suffering. Male Sprague Dawley rats (300–350 g) were anesthetized, skull exposed, and then unilaterally injected in the substantia nigra (SN) with rAAV2/8 expressing human α-synuclein as previously described [23]. Each rat was injected with 2 µL of rAAV2/8 (1.12×10^{10} viral genomes) at 0.4 µL/min using a microinjection pump (Stoelting Co., Wood Dale, IL) with 10 µL Hamilton syringe and 33-gauge needle. After injection the syringe remained in situ for 5 minutes before withdrawal. The scalp was sutured and animals were monitored until fully recovered.

Four days following recovery from surgery, rats began receiving drug or vehicle (0.5% methylcellulose) by oral gavage on a biweekly basis. Figure 1 illustrates the experimental paradigm and structures for each compound. Drug groups included SNX-0723 (PF-04924868) at 10 mg/kg and SNX-9114 (PF-04944733) at 1.5 mg/kg and 3 mg/kg. All rats were weighed routinely prior to surgery, and then at each treatment session for the duration of the experiment. Rats treated with 10 mg/kg SNX-0723 showed toxicity characterized by weight loss and failure to thrive, thus mid-treatment the dose was reduced to 6 mg/kg dose (see results).

Tissue Preparation and Immunohistochemistry

Eight weeks post-injection, rats were deeply anesthetized and transcardially perfused with cold 0.01M phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS. Brains from a subset of animals were harvested fresh, without fixation, and the cortex, striatum, and midbrain quickly dissected on ice, snap-frozen in isopentane, and kept at −80°C for use in biochemical analyses. Perfused brains were postfixed 24 hours, then cryoprotected in 30% sucrose/PBS, and serially sectioned at 40 µm with a sliding microtome. For immunohistochemistry, free-floating sections were rinsed with PBS, then treated with endogenous peroxidase inhibitor (10% methanol and 3% H_2O_2), permeablized with 0.3% Triton X-100 in PBS, and blocked in 5% normal goat serum. Coronal sections through the striatum and SN were immunostained with primary antibodies to TH (1:10,000 dilution; Millipore, Billerica, MA) or α-synuclein LB509 (1:1000 dilution; Zymed Laboratories, Inc., San Francisco, CA) overnight at 4°C. After rinsing, immunostaining was visualized with biotinylated secondary, followed by avidin-biotin (Vectastain Elite Kit), and 3,3′-diaminobenzidine reaction. Immunostained sections were washed and mounted on Superfrost slides, and then counterstained with 0.05% cresyl violet per standard protocols and coverslipped (Permount, Sigma Chemicals).

Microscopy and Stereology

Immunostained sections were viewed using an Olympus BX51 microscope, and photomicrographs were taken with Olympus DP70 digital camera and adjusted for suitable contrast and brightness. Cases in which the AAV injection was improperly placed in the SN (missed target) and poor expression of α-synuclein in the nigrostriatal system were excluded from analyses. Nigrostriatal cell loss was assessed using unbiased stereology according to the optical fractionator principle [27] as previously described [23]. The examiner was blinded to treatment group. Cell counts included the injected side compared to the uninjected contralateral SN as control. At least 8 sections (240 µm apart) though the SN for each case were analyzed and counted using the Olympus CAST Stereology System. Sampling frequency was sufficient for a coefficient of error of less than 0.1.

Immunoblotting

Striatal and midbrain tissues were separately suspended in 8×volume/wet weight tissue of lysis buffer (50 mM Tris-HCL, pH 7.4; 175 mM NaCl; 5 mM EDTA, pH 8.0; and protease inhibitor, Roche Inc.) and homogenized on ice for 10–15 seconds with Teflon pestle. A 100 µL aliquot of this tissue suspension was removed for HPLC and treated with 100 mM H_3PO_4 plus 100 µM methyldopa (internal standard for HPLC recovery). Each sample was centrifuged for 15 minutes at 4°C, filtered (0.22 µm Spin-X filter, Corning, NY), and then 1% Triton X-100 added to the non-HPLC lysate. Lysates were then centrifuged for 60 min at

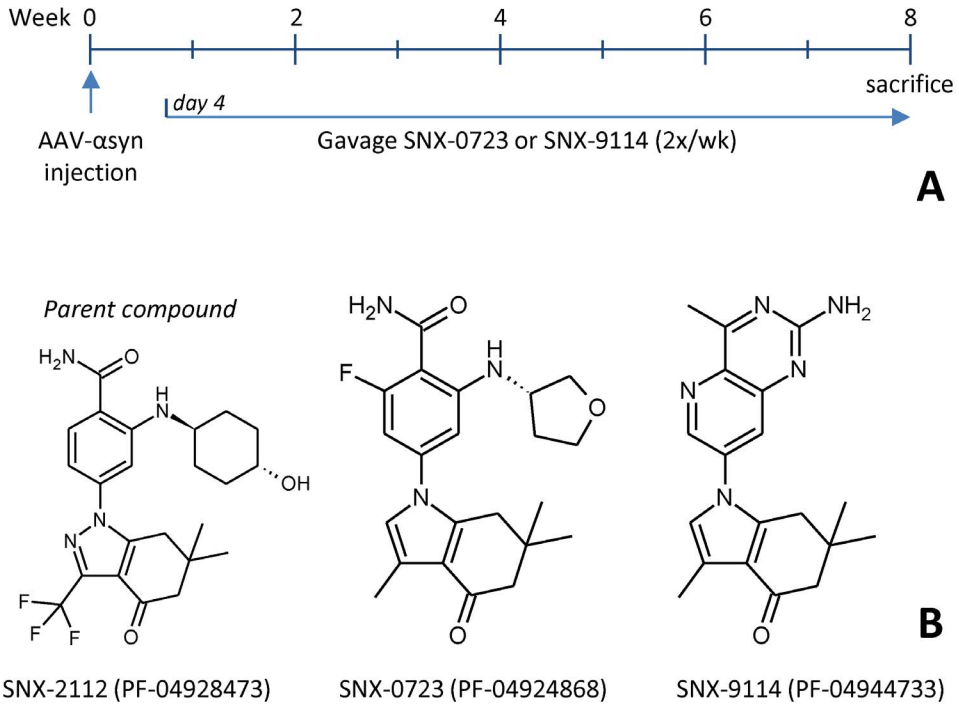

Figure 1. Study paradigm and structure of compounds. A) Illustrates the study paradigm and timecourse for drug testing in animals. **B**) Shows the structures of parent and the two derivative SNX compounds used in experiments.
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4°C to collect the triton-X insoluble fraction. Triton-soluble lysate was separated and the insoluble pellet resuspended in 2% SDS-containing lysis buffer (Triton-insoluble fraction), then sonicated for 10 s. Protein concentration for each lysate was determined by BCA assay. Samples were separated on a 4–12% Bis-Tris NuPage pre-cast gel (Invitrogen) with MES buffer, transferred to PVDF, and immunoblotted for Hsp70 (rabbit anti-Hsp70, Stressgen), tyrosine hydroxylase (TH; mouse TH-2 antibody, Sigma), or α-synuclein (mouse Syn1, BD Transduction Laboratories, or Syn (LB509) antibody, Zymed). All blots were immunostained for GAPDH or actin as loading control. Immunoblotted α-synuclein, TH and GAPDH were detected with secondary antibody conjugated to HRP and reacted with ECL (GE Healthcare), per protocol. Films were digitally scanned and analyzed with ImageJ software (NIH). TH and α-synuclein content for each sample was normalized to loading control.

Hsp70 ELISA

Quantitative analysis of Hsp70 levels in rat cortical (or striatal) tissues after treatment with Hsp90 inhibitors was performed using ELISA (Stressgen, Ann Arbor, MI, USA) according to the manufacturer's instructions and similar to that detailed by Danzer et al. [10]. Hsp70 concentrations in tissues were determined by generating a standard curve with calibrated Hsp70 protein standard and then interpolating absorbance readings using fitting software (Graph Pad 5.0).

Dopamine Content

Striatal tissues were thawed, weighed, homogenized, and mixed in lysis buffer with methyldopa added as an internal control as described above. Dopamine (DA) and 3,4-dihydroxyphenlyacetic acid (DOPAC) were measured by HPLC with electrochemical detection and normalized to protein content per sample [28].

Statistics

All data are expressed as group mean ± SEM. Stereological estimates of nigral TH cell survival were analyzed using one-way ANOVA with Tukey's multiple comparison post-hoc (Prism GraphPad 5.0, San Diego, CA). Dopamine and DOPAC content were analyzed with repeated measures ANOVA and Bonferroni multiple comparisons posthoc and Spearman's correlation. Alpha was 0.05 for all tests.

Results

Preliminary testing of SNX-0723 in rats at doses 10 mg/kg or higher showed lasting induction of Hsp70 in brain at least 24 hours post oral gavage (Figure 2A). The newer compound SNX-9114 likewise demonstrated excellent brain permeability and even greater potency than SNX-0723 in terms of Hsp70 induction. Limited dose-response testing also suggested more prolonged Hsp70 induction, 72 hours or greater, in brain for both Hsp90 inhibitors. Based on these findings we compared the effects of chronic oral treatment of rats with SNX-0723 at 10 mg/kg versus SNX-9114 at 1.5 and 3 mg/kg for 7–8 weeks post injection of AAV-α-synuclein. Western blot analysis of striatal extracts collected 3–4 days post final treatment confirmed a sustained 2-fold increase in Hsp70 induction in SNX-9114 treatment groups compared to vehicle (Figure 2B, C).

Tolerability and Toxicity

Although chronic treatment with SNX-9114 was generally well tolerated, SNX-0723 at 10 mg/kg resulted in toxicity manifest by diarrhea, weight loss, failure to thrive, and early death in 7 of 21 animals. As a result, the dose of SNX-0723 was reduced mid-treatment to 6 mg/kg for all remaining rats in this group. Dose reduction was effective in reducing toxicity, reversing weight loss and mortality. However, rats did not gain weight at the same rate

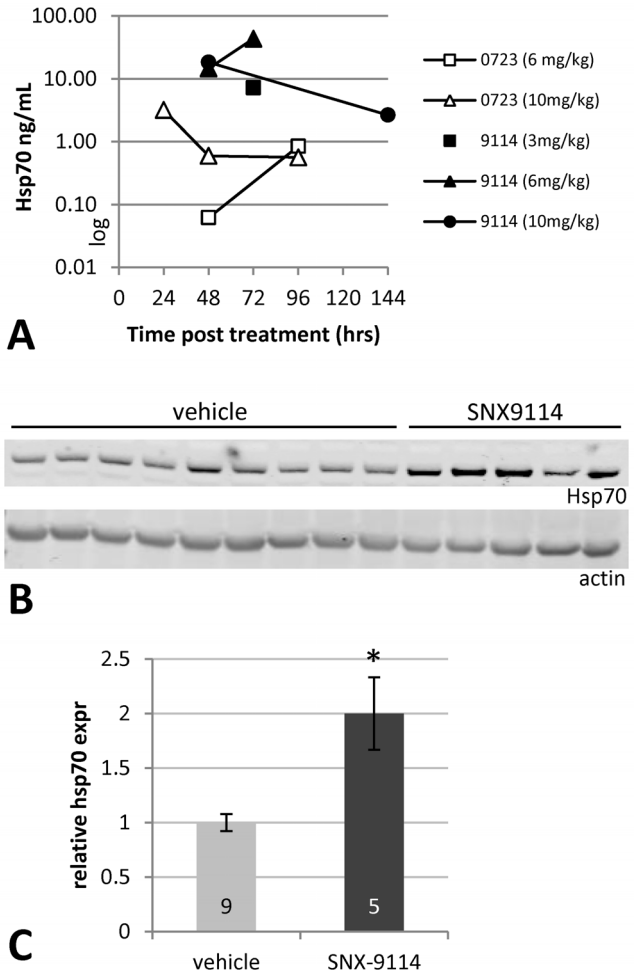

Figure 2. Hsp70 induction in brain. A) Graph of hsp70 ELISA data from cortical tissue lysates after treatment of rats with novel small molecule Hsp90 inhibitors. Tissue was harvested at 1–6 days post treatment and shows sustained hsp70 induction at ≥72 hrs for both SNX-0723 and SNX-9114. **B**) Western blot of striatal tissue homogenates from rats injected with WT α-synuclein and treated with SNX9114 (n = 5) or vehicle (n = 9), immunoblotted for hsp70 and actin as loading control. **C**) Densitometry shows significant (p = 0.037) striatal Hsp70 induction following treatment with SNX-9114.
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as vehicle control animals (Figure 3). Similarly, although rats treated with 3 mg/kg SNX-9114 did not show overt signs of toxicity, weight gains were less than that of control. Halving the SNX-9114 dose to 1.5 mg/kg in a separate treatment group made little difference in weight gain. No overt behavioral changes were observed in either of the groups or treatment regimens.

Effects of Hsp90 Inhibitors on Nigrostriatal Toxicity

Chronic treatment with SNX-0723 or with the more potent SNX-9114 did not rescue nigral dopaminergic neurons from α-synuclein dependent toxicity. Figure 4A-C shows the distribution of TH-immunoreactive cell loss at comparable levels of the SN for both drug and vehicle treatment after viral injection. Stereological counts revealed mean TH cell loss (relative to the contralateral unlesioned SN) of 21.1%±3.7 for vehicle, 21.6%±5.0 for 6–10 mg/kg SNX-0723, and 17.0%±3.2 and 24.1%±4.7for 1.5 and 3 mg/kg doses of SNX-9114, respectively (Figure 4E). Although the lower dose of SNX-9114 appeared to have slightly less TH cell loss, one-way analysis of variance revealed no significant differences among treatment groups ($F[3,31] = 0.42$, $p = 0.39$). Similarly, analysis of striatal TH terminal density as shown in representative cases (Figure 5) demonstrated no differences between drug and vehicle control groups. Likewise, among different treatment groups there was also no apparent change in α-synuclein-positive inclusion-like structures in nigrostriatal terminals or cell bodies.

Hsp90 Inhibitor Effects on Dopamine Terminals

We performed biochemical analysis of striatal DA and its metabolite, DOPAC, to gauge drug effects on nigrostriatal terminal plasticity. Striatal DA and DOPAC measurements ipsilateral (ip) to rAAV α-synuclein injection were normalized to the contralateral (ct) uninjected/unlesioned side within each animal and shown as ratio of ip/ct (Figure 6A). Repeated measures ANOVA demonstrated a significant main effect for drug ($F[3,25] = 7.05$, $p = 0.001$) and interaction with DA and DOPAC measures ($F[3,25] = 3.31$, $p = 0.036$). Vehicle treated animals, as expected, showed (~50%) reduction in striatal DA ipsilateral to rAAV α-synuclein injection with mean DA ratio of 0.51±0.10 relative to that in the contralateral striatum. SNX-0723 at the 6–10 mg/kg dose did not significantly alter striatal DA levels (0.62±0.10) and was similar to vehicle control. However, treatment with SNX-9114 resulted in significant increase and trend toward normalization of striatal DA and DOPAC levels compared to vehicle. DA content for the 1.5 mg/kg dose was 1.04±0.13 ($p = 0.061$) and the 3 mg/kg dose 1.45±0.32 ($p = 0.003$). DOPAC was also significantly increased for SNX-9114 1.5 mg/kg dose, 1.36±0.15 ($p = 0.005$), and likewise showed a non-significant trend for normalization at 3 mg/kg dose, 1.12±0.22 ($p = 0.28$). We also examined an index of DA turnover to DOPAC (DOPAC/DA ratio), which negatively correlated with DA changes, $r_s = -0.67$, $p<0.01$ (Figure 6B). Decreases in DA for control and SNX-0723 at 6–10 mg/kg corresponded to increase in DOPAC/DA ratio (1.57±0.2 and 1.34±0.16, respectively), or turnover. By contrast, in the case of the 3 mg/kg dose of SNX-9114 relative increase in striatal DA resulted in non-significant decrease in DOPAC/DA ratio (0.81±0.09).

Discussion

Modulation of molecular chaperones with small molecule Hsp90 inhibitors has gained recent attention as potential novel therapeutics for parkinsonism and other neurodegenerative disorders that manifest proteinopathy [13,29,30]. We recently reported that novel small molecule Hsp90 inhibitors in a neuroglioma cell model of parkinsonism can reduce formation of toxic dimer/oligomeric species of α-synuclein and prevent cytotoxicity [22]. In the current study, we examined whether chronic treatment with candidate, small molecule Hsp90 inhibitors could protect against α-synuclein-induced nigrostriatal toxicity in a targeted viral model of parkinsonism in the rat. Chronic treatment twice weekly was best tolerated with SNX-9114, but neither SNX-0723 nor SNX-9114 protected against loss of dopaminergic nigrostriatal neurons in our model. Several possibilities may explain these results including length of treatment, onset of therapy, and inter-animal variability. Longer incubation with AAV-synuclein (12 vs 8 weeks, personal observation) can result in greater dopamine cell loss and, combined with chronic Hsp90 inhibitor therapy, might have increased our ability to detect potential differences between vehicle and drug groups. Although we started treatment early–4 days post viral injection when viral transgene expression is only beginning–pretreatment before AAV

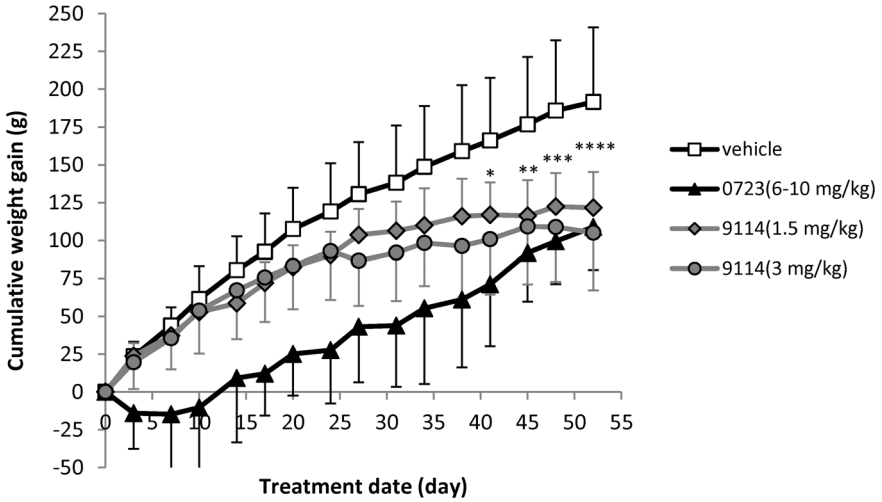

Figure 3. Graph of cumulative weight gain for treatment groups. SNX-0723 caused the most toxicity, weight loss, and failure to thrive at the higher dose of 10 mg/kg. All treatment groups gained weight at lower doses, including SNX-9114, but not at the rate of vehicle control (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ for comparison of vehicle and 9114 at 1.5 mg/kg; 2-way ANOVA with Bonferroni correction). (n = 14, 14, 12, 10, and 14 for vehicle, 0723 [3mg/kg], 0723 [6–10 mg/kg], 9114 [1.5 mg/kg] and 9114 [3 mg/kg], respectively).
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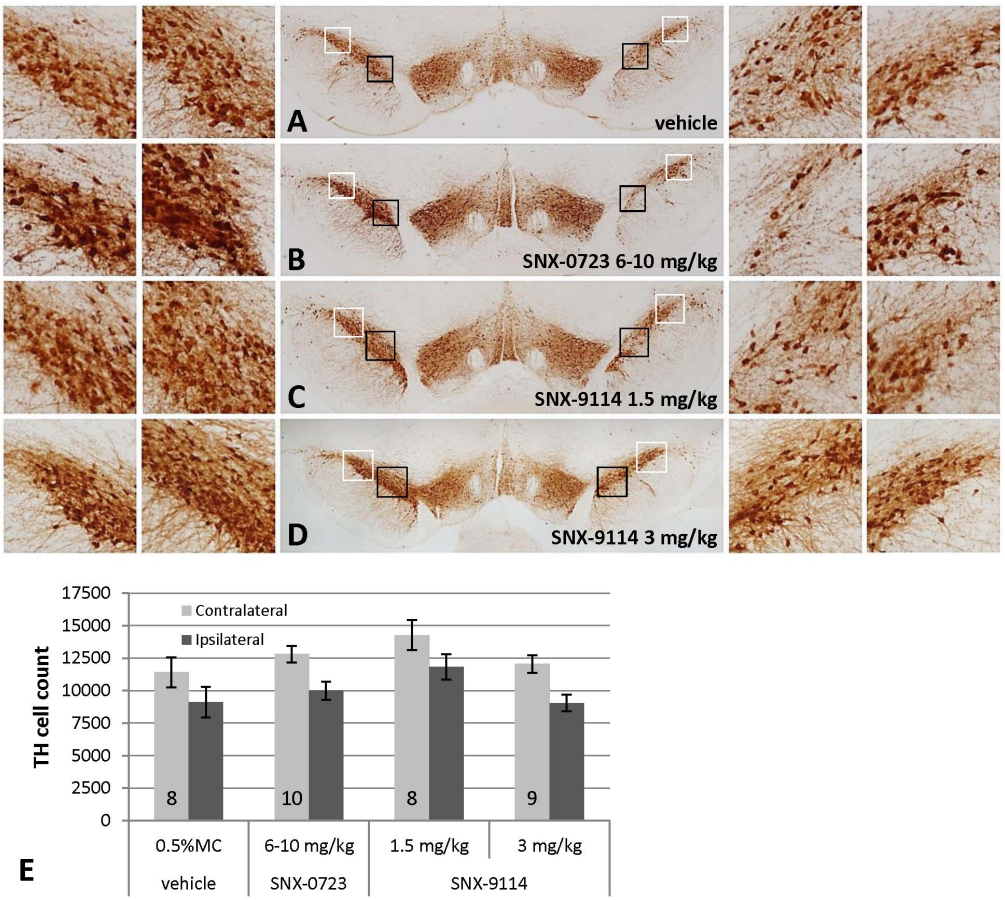

Figure 4. Comparison of higher dose SNX-0723 and SNX-9114 effects on nigrostriatal toxicity. A–D) Photos show low power images of injected SN (right) and contralateral uninjected side (left) for each drug treatment group. Black (medial) and white (lateral) squares indicate regions of interest for higher magnification photos shown. There is modest cell loss on the side of the lesion for all treatments. **E**) Graph of stereological counts (mean ±SEM) of TH-positive cells in the SN ipsilateral and contralateral to AAV-α-synuclein lesion. Numbers at base of bars indicate N for each group. Analysis of variance revealed no significant differences among treatment groups.
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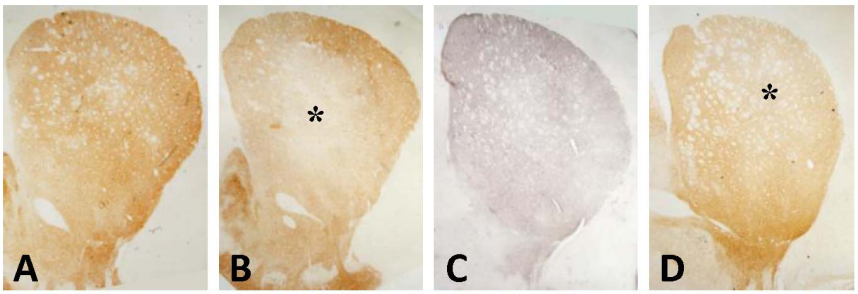

Figure 5. Illustration of drug effects on nigrostriatal terminal density for SNX-9114, 3 mg/kg dose. The representative photos show the distribution of TH+ terminals in the striatum contralateral and ipsilateral to AAV-α-synuclein injection in panels **A** and **B,** respectively. **C)** α-Synuclein-positive nigrostriatal terminals ipsilateral to AAV injection in same case. **D**) Photo of TH+ terminals in striatum from animal treated with lower dose SNX-9114, 1.5 mg/kg. *Marks region of TH terminal loss due to α-synuclein toxicity.
doi:10.1371/journal.pone.0086048.g005

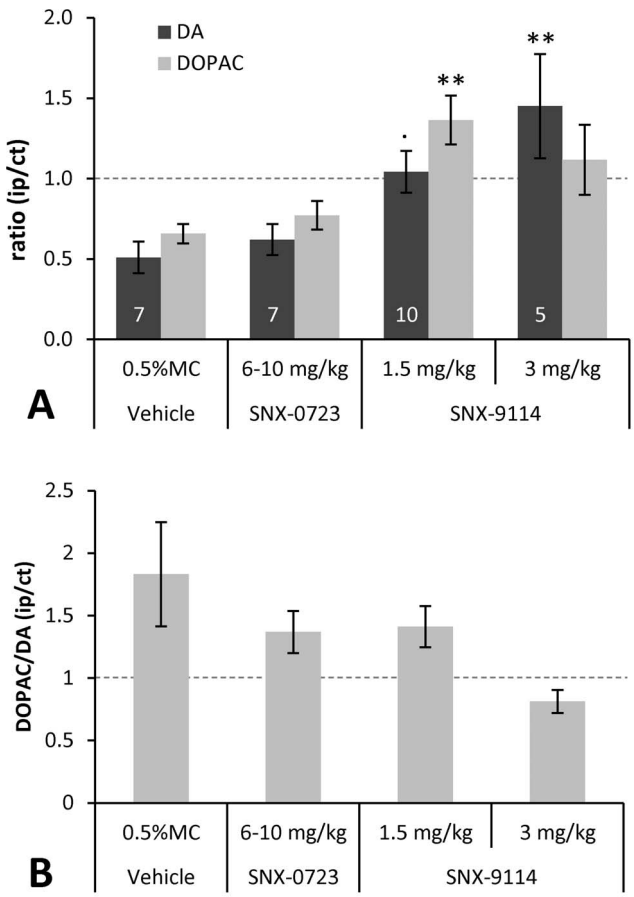

Figure 6. Striatal DA and DOPAC content and turnover. A) Graph shows the mean (±SEM) DA or DOPAC content in the striatum ipsilateral (ip) to AAV-α-synuclein injection normalized to the contralateral (ct) uninjected side (ratio ip/ct). Repeated measures ANOVA (DA and DOPAC) showed a main effect for drug ($F[3,25] = 7.05$, $p = 0.001$) and interaction with DA metabolites ($F[3,25] = 3.31$, $p = 0.036$). DA levels were significantly increased for SNX-9114 at both 1.5 ($p = 0.061$) and 3 ($p = 0.003$) mg/kg doses compared to vehicle, whereas no change for SNX-0723. DOPAC levels increased significantly only for SNX-9114 at 1.5 mg/kg ($p = 0.005$), but appeared also to trend toward normal for the higher dose. N for each group is noted at base of each bar (7, 7, 10, 5, respectively). **B**) Graph of DA turnover (DOPAC/DA ratio, normalized to contralateral control) for each case shows an inverse correlation between DA level and rate of turnover. ·$p = <0.1$, **$p<0.01$.
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injection may be required in our model to prevent dopamine cell loss in the rat SN as seen in prior cell studies [22]. Variability in this model and treatment paradigm likewise could have contributed to lack of findings, indicating need for greater animal numbers to the increase the power of our observations.

Although Hsp70 expression or induction (i.e., via Hsp 90 inhibition) has been shown to reduce α-synuclein dimer/oligomers and cytotoxicity in cell models [10,12,31,32], few studies have examined the effects of increased Hsp70 on α-synuclein toxicity in animal models. In *Drosophila* Hsp70 expression has been shown to reduce dopaminergic neuronal loss associated with α-synuclein [11]. Crossing Hsp70 expressing mice with transgenic mice that express human wild-type α-synuclein (line D), we subsequently demonstrated that Hsp70 specifically reduces "toxic" high-molecular weight α-synuclein species [9]. In contrast, Shimsheck et al. (2010) examined transgenic mice co-expressing both human A53T mutant α-synuclein and Hsp70(HspA1A) under the control of the Thy1 promoter and found that mice overexpressing Hsp70 actually performed worse on behavioral tests than single transgenic α-synuclein(A53T) mice [33]. Moreover, Hsp70 overexpression did not cause change in α-synuclein expression, oligomers, phosphorylation, or localization in brain. These findings are difficult to explain, but possibilities include inadequate level of Hsp70 expression, non-functional Hsp70, or lack of other co-chaperones such as Hsp40 or Hsp90 which enhance Hsp70 ATPase activity [34]. Differences in interaction between Hsp70 and wild-type vs A53T α-synuclein may also contribute but remain unclear. Besides Hsp70 other heat shock proteins may be (more) effective, such as Hsp104 which when tested in a rat model of α-synuclein overexpression reduced dopaminergic cell loss and phosphorylated α-synuclein-containing inclusions [35]. In vitro Hsp27 expression has also been shown to have more potent effect than Hsp70 on toxicity associated with mutant and wild-type α-synuclein [36]. Recent studies by Daturpalli et al. (2013) suggest also that Hsp90 itself interacts with oligomeric α-synuclein and can inhibit fibril formation and α-synuclein toxicity in SHSY5Y cells [37]. Together these data indicate need for further study of heat shock protein effects on α-synuclein in both cell and animal models.

Despite the lack of apparent rescue of nigrostriatal dopamine cells, we observed a significant drug effect on striatal DA content and metabolism. In animals treated with SNX-9114 striatal DA and DOPAC levels increased and "normalized," suggesting a possible effect on the remaining nigrostriatal terminals and neurochemical plasticity. These preliminary findings are potentially significant as restoration of dopamine content in the striatum improves behavioral deficits in PD models and overall function in

PD patients [38,39]. Potential mechanisms for the observed increase in striatal DA include increased TH activity or L-DOPA (L-dihydroxyphenylalanine) supply, decrease in monoamine oxidase B activity, or increased terminal DA reuptake. Compensatory mechanisms for nigrostriatal injury are well-established and residual striatal terminals can compensate for nearly 80% loss of DA innervation [38,40]. Recent data, however, suggests that α-synuclein expression negatively regulates TH activity and can affect dopaminergic neurotransmission [41]. Results in vehicle control animals are consistent with these findings and show reduced striatal DA without evidence of compensation despite relative small nigrostriatal lesion (~21% TH cell loss). However, the cause of increase or normalization of DA levels with SNX-9114 is less clear. Although we did not measure TH activity, striatal levels of TH for treated and control animals appeared similarly reduced due to AAV-α-synuclein lesion and there was no evidence of TH terminal sprouting as seen previously in other partial lesion models [40,42]. While Hsp70 induction has been shown to protect dopaminergic neurons against toxic insult, including α-synuclein, very little is known about the potential effects on DA production (i.e. TH activity) or metabolism [43,44]. Further studies are needed to evaluate possible neuromodulatory effects of small molecular Hsp90 inhibitors and Hsp70 induction on dopaminergic neurons.

A major limitation of Hsp90 inhibitor therapy unfortunately has been toxicity, which was also found for the drugs used in this study [13]. Modifications to geldanamycin leading to development of the analogues 17-AGG and 17-DMAG were initially purported to reduce toxicity, mainly hepatic, and increase potency as well as brain penetration [6,17]. Clinical trials of these compounds primarily for cancer therapy have shown some promise, but significant concerns about hepatotoxicity and delivery issues remain, limiting their use in particular for non-oncology indications [18,19]. Recent efforts have focused on developing novel small molecule Hsp90 inhibitors, such as those studied herein which potently inhibit Hsp90, cross the blood-brain barrier, and are orally bioavailable [20,21]. Our initial studies in rodents demonstrate that candidate drugs, administered orally, were brain permeable at concentrations used and produced lasting induction of Hsp70 in brain tissue. However, SNX-0723 given chronically at 10 mg/kg caused animals to loose significant weight, fail to thrive, and die, forcing decrease in the dose to 6 mg/kg which was better tolerated. Although the more potent SNX-9114 did not cause overt toxicity at either dose used, rats still did not gain weight at rates equivalent to vehicle treated animals. While SNX-9114 induced Hsp70 in brains, it too had an insignificant neuroprotective effect on synucleinopathy. It is tempting to speculate whether Hsp70 induction in brain had a causal relationship to weight loss/failure-to-thrive in animals, but based on prior studies it is more likely that our candidate drugs caused peripheral toxicity (i.e., hepatic, gastrointestinal) [29,45]. No studies to our knowledge so far have linked Hsp70 (or Hsp90) to weight homeostasis or metabolism. Further studies are needed to elucidate the source of toxicity for future trials.

To date clinical trials for Hsp90 inhibitors have primarily been limited to cancer therapy, based on their selectivity for tumor cells, modulation of Hsp90 function, and binding of client proteins [29,46]. Kamal *et al.* have suggested that the geldanamycin derivative, 17-AAG, preferentially binds Hsp90 when it is part of a multi-chaperone complex, including co-chaperones and client protein [47,48]. Although it is unclear if novel small molecule Hsp90 inhibitors such as those used here function similarly, such selectivity may provide similar advantage for use in neurodegenerative disorders particularly due to the probable need for long-term, chronic therapy, relative to that in cancer. Our findings, however, indicate that Hsp70 induction in brain was widespread rather than limited to tissues affected by viral α-synuclein expression. Though potentially concerning, such effects in brain may actually be advantageous. Heat shock protein induction (i.e., stress response) by Hsp90 inhibition has been shown to have purported neuroprotective effects in a variety of neurodegenerative models including Huntington's disease [7], spinocerebellar ataxias [6], tauopathies [8], and parkinsonism [10] in which pathology spreads. Neuroprotective effects of Hsp70 induction in particular include reduction in aggregate ("toxic" oligomer) formation, cellular toxicity, and apoptosis [9,10,49]. Thus, targeting Hsp90 and augmenting the cellular response to stressors may still be a reasonable therapeutic approach for neurodegenerative diseases.

This study represents a first attempt to examine the ability of novel small molecule Hsp90 inhibitors to protect against α-synuclein dependent nigrostriatal toxicity in mammalian model of PD. Compared to vehicle neither compound tested protected against nigral TH-cell loss; however, our results suggest possible nigrostriatal terminal effects with normalization of DA content and turnover in striatum. These results are significant as restoration of DA in the brain is an aim of current therapeutics in Parkinson disease. Although the mechanism of nigrostriatal dopamine restoration remains unclear, these findings suggest that Hsp90 inhibition may represent a potential novel therapeutic approach to Parkinson disease and related disorders. Further study of these novel small molecule Hsp90 inhibitors is warranted and must also address toxicity concerns for future trials in neurodegenerative disease.

Acknowledgments

The authors dedicate this manuscript to the memory of Laura Kibuuka. We thank C.D.H. and W.H. for providing the drugs which are covered by Pfizer patents WO 2008130879, WO 2006091963, and WO 2008024977.

Author Contributions

Conceived and designed the experiments: NRM HD PJM. Performed the experiments: NRM HD LK DEF KMD MD. Analyzed the data: NRM HD LK DEF PJM. Contributed reagents/materials/analysis tools: ZF CAD MAS WH. Wrote the paper: NRM HD PJM.

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REVIEW

Treatment of Parkinson's Disease: What's in the Non-dopaminergic Pipeline?

Albert Y. Hung · Michael A. Schwarzschild

Abstract Dopamine depletion resulting from degeneration of nigrostriatal dopaminergic neurons is the primary neurochemical basis of the motor symptoms of Parkinson's disease (PD). While dopaminergic replacement strategies are effective in ameliorating these symptoms early in the disease process, more advanced stages of PD are associated with the development of treatment-related motor complications and dopamine-resistant symptoms. Other neurotransmitter and neuromodulator systems are expressed in the basal ganglia and contribute to the extrapyramidal refinement of motor function. Furthermore, neuropathological studies suggest that they are also affected by the neurodegenerative process. These non-dopaminergic systems provide potential targets for treatment of motor fluctuations, levodopa-induced dyskinesias, and difficulty with gait and balance. This review summarizes recent advances in the clinical development of novel pharmacological approaches for treatment of PD motor symptoms. Although the non-dopaminergic pipeline has been slow to yield new drugs, further development will likely result in improved treatments for PD symptoms that are induced by or resistant to dopamine replacement.

Keywords Parkinson's disease · Non-dopaminergic · Dyskinesias · Motor fluctuations · Glutamate · Adenosine

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is characterized clinically by the classical motor symptoms of bradykinesia, rigidity, and resting tremor. These symptoms are primarily caused by the selective loss of dopaminergic neurons in the substantia nigra pars compacta, which results in decreased levels of dopamine in the striatum. Dopamine replacement strategies have been the mainstay of treatment for motor symptoms of PD, and nearly 50 years since its introduction, levodopa (the precursor of dopamine) remains the most effective treatment. However, despite its beneficial effects on motor function, dopaminergic therapy has significant limitations, making development of other therapeutic approaches targeting non-dopaminergic pathways a priority [1]. First, neither levodopa nor dopamine agonists have been demonstrated to slow the progression of nigrostriatal cell loss. Second, while initially successful in ameliorating motor symptoms, long-term treatment with levodopa is complicated by the onset of motor fluctuations (with alternating periods of mobility and relative immobility) and involuntary dyskinesias. Last, symptoms that develop at later stages of PD, both motor (e.g., postural instability and freezing of gait) and non-motor, are frequently not responsive to dopaminergic treatments. These symptoms are likely to be caused by the degeneration of neurons in other parts of the nervous system as a result of the same disease process that affects the nigrostriatal system [2].

In this review, we discuss potential non-dopaminergic approaches to treatment of PD symptoms. Multiple neurotransmitters are recognized to play a role in modulating the basal ganglia and other neural circuits thought to be involved in PD. We will focus primarily on neurotransmitter targets in which there have been therapeutic advances in targeting motor symptoms. Agents targeting non-dopaminergic pathways are also being actively explored for treatment of non-motor symptoms.

Neurotransmitter Diversity in the Basal Ganglia and Motor Control

To understand the potential use of pharmacologic agents targeting non-dopaminergic pathways, it is helpful to briefly

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review the role of these neurotransmitter systems in regulating motor function [3]. In the classic model of basal ganglia organization (Fig. 1), the cerebral cortex sends excitatory glutamatergic inputs to the striatum. Dopamine, via the nigrostriatal pathway, modulates these inputs, either through an excitatory effect on a subpopulation of striatal neurons that contain gamma-aminobutyric acid (GABA) and substance P (direct pathway), or through an inhibitory effect on a separate subpopulation of neurons that co-express GABA and enkephalin (indirect pathway). The effects on the direct and indirect pathways are mediated by dopamine binding to D1 and D2 receptors, respectively, both of which are highly expressed in the striatum (Fig. 2). In the direct pathway, striatal neurons send inhibitory GABAergic inputs directly to the output nuclei of the basal ganglia, the globus pallidus pars interna (GPi) and substantia nigra pars reticulata (SNr), which then send GABAergic fibers to ventral thalamic nuclei. In contrast, axons from striatofugal neurons in the indirect pathway form GABAergic synapses with cells in the globus pallidus pars externa, which then send GABAergic projections to the subthalamic nucleus (STN). The STN then uses glutamate to modulate basal ganglia output from the GPi/SNr. This classic model suggests that dopamine regulates basal ganglia activity by balancing opposing effects on the direct and indirect pathways. Loss of striatal dopamine in PD disrupts this balance, producing a hypokinetic (parkinsonian) state. In contrast, subsequent treatment with dopaminergic agents predisposes to hyperkinetic (dyskinesia) responses. While this model is

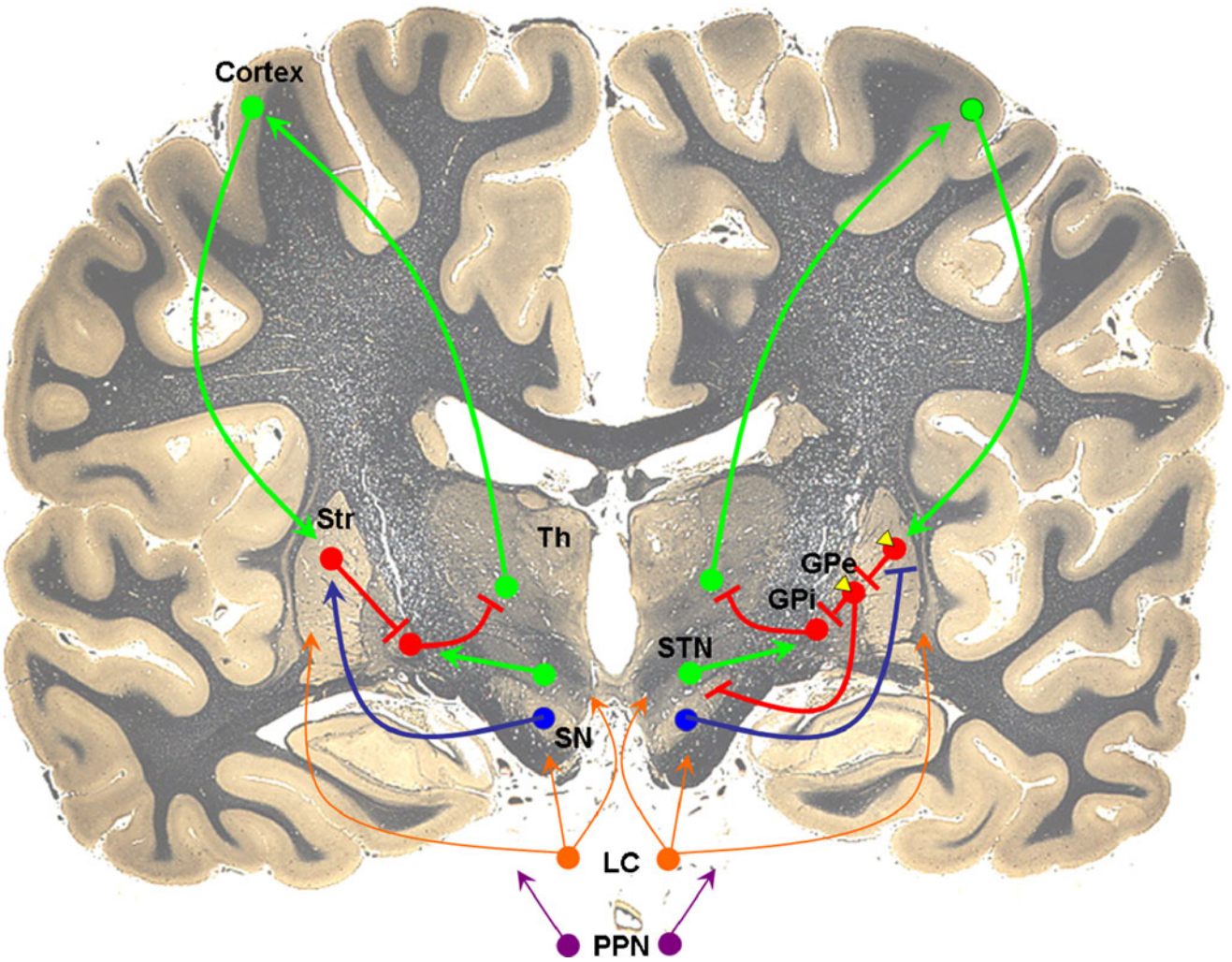

Fig. 1 Neurotransmitter systems involved in basal ganglia circuitry. Excitatory glutamatergic efferents (green) from cortex project to gamma-aminobutyric acid (GABA)ergic (red) striatal neurons. In the direct pathway (left), striatal neurons receive excitatory dopaminergic inputs (blue) from substantia nigra and project directly to globus pallidus interna (GPi). In the indirect pathway (right), dopamine inhibits striatal GABAergic output to the globus pallidus externa (GPe), which then projects to GPi. Adenosine A_{2A} receptors (yellow) are localized to dopamine D2 receptor-containing cells in the indirect pathway. Noradrenergic and cholinergic efferents from the locus coeruleus (orange) and pedunculopontine nucleus (purple), respectively, project widely to multiple brain regions, including cortex and basal ganglia. The coronal brain image is adapted with permission from http://www.brains.rad.msu.edu and http://brainmuseum.org, supported by the US National Science Foundation

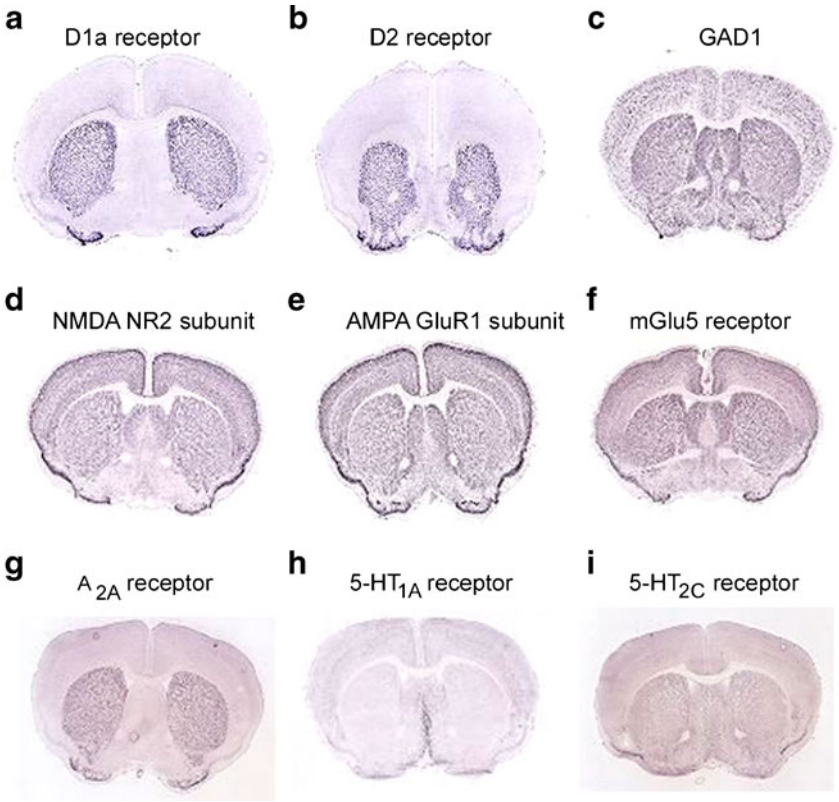

Fig. 2 Expression patterns of neurotransmitter systems in the rodent brain. Dopamine D1 and D2 receptors and adenosine A_{2A} receptors are localized and highly expressed in the striatum, while glutamic acid decarboxylase [GAD, present in gamma-aminobutryic acid (GABA)ergic neurons], N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA), and metabotropic glutamate receptor (mGlu5) subunits, and serotonin (5-HT) receptor subtypes are not concentrated in specific brain regions. In situ hybridization images are obtained from the Allen brain atlas (www.brain-map.org)

useful in accounting for some of the phenomenology associated with PD, basal ganglia circuitry is likely to be more complicated. For example, a recent rodent study suggests that both direct and indirect pathways are concurrently activated during initiation of action [4].

Glutamate receptors are expressed at high levels in the striatum. However, unlike dopamine receptors (which are highly enriched in the basal ganglia), they are present at high density throughout the brain (Fig. 2). GABA, which is synthesized from glutamate by the enzyme glutamic acid decarboxylase (GAD), is also expressed widely in the central nervous system (CNS). Given their primary role in basal ganglia circuitry, these neurotransmitter systems are potentially attractive targets to treat parkinsonian symptoms. However, their lack of regional specificity raises the potential challenge of side effects from actions on other brain regions.

Other neurotransmitters have also been implicated in the regulation of basal ganglia function. Adenosine is a purine nucleoside that acts to modulate synaptic function in the CNS. Its action is mediated by 4 G-protein-coupled receptor subtypes: A_1, A_{2A}, A_{2B}, and A_3. Of these, the A_{2A} receptor has received considerable attention as a potential treatment target because, like dopamine receptors, its expression is highly enriched in the striatum (Fig. 2) [5, 6]. Alterations in the serotonergic system have also been recognized in PD [7]. Of the 14 subtypes of serotonin (5-HT) receptors [8], multiple subtypes, including 5-HT_{1A} and 5-HT_{2C} receptors, are present in striatal neurons (Fig. 2). Serotonergic inputs from the raphe nuclei form widespread connections throughout the brain, including the substantia nigra, striatum, globus pallidus, STN, thalamus, and cortex.

Neuropathological studies have suggested that neurodegeneration in PD is not restricted to dopaminergic neurons and the basal ganglia. According to the Braak staging system [2], inclusion bodies containing α-synuclein are found in caudal brainstem nuclei (stage 1) prior to involvement of the substantia nigra (stage 3). At stage 2, 5-HT-producing raphe nuclei neurons are affected, as are projection neurons in the locus coeruleus that produce noradrenaline. At later stages (through stage 6), acetylcholine (ACh)-producing neurons in the pedunculopontine tegmental nucleus and neocortex also undergo degeneration. The diversity of affected neurotransmitter systems yields a number of symptoms that may only respond to adjunct non-dopaminergic therapies.

Symptomatic Treatment and Motor Fluctuations

The presence of multiple neurotransmitters modulating the basal ganglia circuitry that coordinates movement suggests that non-dopaminergic strategies may be helpful in treating motor symptoms. These approaches offer potential advantages, including providing antiparkinsonian benefits either as monotherapy or in combination with dopamine replacement,

allowing reduction in dose of dopaminergic agents to ameliorate treatment-related side effects, or directly reducing motor fluctuations and/or dyskinesias associated with chronic levodopa use.

Adenosine

Adenosine A_{2A} receptors are localized to dendrites, cell bodies, and axon terminals of GABAergic striatopallidal neurons of the indirect pathway, in close association with dopamine D_2 receptors [9–11]. By binding to A_{2A} receptors, adenosine activates striatopallidal neurons, opposing the inhibitory effects mediated by D2 receptor binding [12, 13]. These findings suggest that blockade of A_{2A} receptors should inhibit the excessive activity of the indirect pathway that results from dopamine depletion. Indeed, in rodent and non-human primate models of PD, A_{2A} antagonists consistently reversed parkinsonian deficits without development of tolerance to prolonged treatment [14]. The preclinical data have motivated multiple clinical trials investigating whether these agents are effective in treating PD symptoms (Table 1).

Among A_{2A} antagonists that have been investigated clinically, istradefylline (KW-6002) has been studied most extensively. In a phase II randomized clinical trial, istradefylline did not improve motor function when used as monotherapy [15]. However, multiple phase II clinical trials in levodopa-treated PD patients with motor fluctuations demonstrated a significant reduction in off time [16–20]. Several of these trials demonstrated an increase in on time with dyskinesias, although they were not troublesome and did not impair mobility [17–19]. A long-term, open-label study showed persistent improvement in off time over a 52-week treatment period, suggesting a sustained symptomatic benefit [21]. However, despite the initial optimism based on the early studies, subsequent phase III clinical trials have yielded conflicting results. Two studies demonstrated a significant reduction in daily off time with an increased incidence of dyskinesias [22, 23], but istradefylline did not affect off time in another trial [24]. In the latter study, motor function in the on state was improved compared with placebo, and a large placebo response may account for the negative effect on off time [24]. Although the US Food and Drug Administration issued a not approvable letter for istradefylline based on available data in 2008 [25], the drug was later approved for use in Japan as adjunctive treatment for PD [26], and phase III clinical development recently resumed in North America [27].

More recently, preladenant, a second-generation A_{2A} antagonist with higher affinity and greater selectivity, had been moving through the therapeutic pipeline. In a phase II, dose-finding, 12-week randomized, placebo-controlled trial, preladenant at a dose of 5 mg and 10 mg twice daily was well-tolerated and reduced off time without increasing on time with troublesome dyskinesias [28]. In a 36-week open-label extension study, the drug similarly provided a reduction in off time, but with an increased incidence of dyskinesias (33 % *vs* 9 % in the randomized study) [29]. Three separate phase III randomized, controlled clinical trials have been ongoing, 2 assessing preladenant when added to levodopa in patient with moderate-to-severe PD, and another as monotherapy in early PD. Results have not been presented or published, but a press release from the manufacturer [30] indicated that initial review did not show evidence of efficacy; as a result, extension studies were discontinued and there are no plans to pursue regulatory filings.

A phase IIb randomized clinical trial investigating the safety and efficacy of the A_{2A} antagonist tozadenant (SYN115) to treat end-of-dose wearing off in 420 patients with moderate-to-severe PD patients has been completed, and a preliminary communication reported good tolerability and significant reduction in off time [31]. A previous smaller clinical study of tozadenant in PD patients provided functional magnetic resonance imaging evidence that the drug enters the CNS and engages its putative target of striatopallidal adenosine A_{2A} receptors to reduce the inhibitory influence of the indirect pathway on motor function [32].

Lastly, it is worth noting that the non-specific adenosine receptor antagonist caffeine, likely acting by blocking striatal A_{2A} receptors [33], has recently demonstrated evidence of significant antiparkinsonian actions in a randomized clinical trial. Although the study by Postuma et al. [34] was designed primarily to investigate potential alerting effects, they observed a reduction in Unified Parkinson's Disease Rating Scale score comparable to that with more specific A_{2A} antagonists and are now pursuing a long-term phase III study to investigate potential disease-modifying benefits, as well as to possibly confirm short-term motor benefits. Convergent epidemiological and laboratory animal data also support the neuroprotective potential of A_{2A} antagonists, including caffeine, in PD [35]. Similarly, clinical, pathological, imaging, and laboratory findings have suggested these agents may help prevent the development of dyskinesias in PD [36–39].

GABA: Glutamic Acid Decarboxylase Gene Therapy

In PD, loss of dopaminergic neurons in the nigrostriatal pathway and reduction of striatal dopamine levels results in disinhibition of the subthalamic nucleus that causes parkinsonian symptoms. The enzyme GAD converts glutamate into GABA, the major inhibitory neurotransmitter in the brain. GAD gene transfer using an adeno-associated virus (AAV) has been explored as an approach

Table 1 Non-dopaminergic therapies for motor symptoms of Parkinson's disease: Results from clinical trials

Mechanism	Drug	Phase	Use	n	Dose	Duration	Primary Outcome	Result	Ref
SYMPTOMATIC TREATMENT AND MOTOR FLUCTUATIONS									
Adenosine									
A_{2A} antagonist	Istradefylline (KW-6002)	II	Mono	176	40 mg/day	12 weeks	Change from baseline in UPDRS motor score	Negative	[15]
			Adjunct	15	40 or 80 mg/day	6 weeks	Duration of L-dopa effect	Positive	[16]
			Adjunct	83	5/10/20 or 10/20/40 mg/day	12 weeks	Reduction in off time	Positive	[17]
			Adjunct	395	20 and 60 mg/day	12 weeks	Reduction in off time	Positive	[18]
			Adjunct	196	40 mg/day	12 weeks	Reduction in off time	Positive	[19]
			Adjunct	363	20 or 40 mg/day	12 weeks	Reduction in off time	Positive	[20]
		III	Adjunct	231	20 mg/day	12 weeks	Reduction in off time	Positive	[22]
			Adjunct	373	20 or 40 mg/day	12 weeks	Reduction in off time	Positive	[23]
			Adjunct	584	10, 20, or 40 mg/day	12 weeks	Reduction in off time	Negative	[24]
	Preladenant	II	Adjunct	253	1, 2, 5, or 10 twice daily	12 weeks	Reduction in off time	Positive (for 5, 10 mg doses)	[28]
	Tozadenant	II	Adjunct	420	60, 120, 180, or 240 mg twice daily	12 weeks	Reduction in off time	Positive (for 120, 180 mg doses)	[31]
GABA									
GAD gene therapy	AAV2–GAD	II	Adjunct	45	Bilateral AAV2–GAD delivery	6 months	Change from baseline in off state UPDRS motor score	Positive	[42]
Serotonin									
5-HT_{1A} agonist	Pardoprunox	II	Mono	139	9-45 mg/day	3 weeks	Change from baseline in UPDRS motor score	Positive	[49]
		III	Mono	468	6, 12, or 12–42 mg/day	24 weeks	Change from baseline in UPDRS motor score	Positive (high dropout rate)	[50]
			Mono	334	12–42 mg/day (*vs* pramipexole)	24 weeks	Change from baseline in UPDRS motor score	Positive (high dropout rate)	[50]
		III	Adjunct	295	12–42 mg/day	12 weeks	Reduction in off time	Positive (high dropout rate)	[51]
LEVODOPA-INDUCED DYSKINESIAS									
Glutamate									
NMDA receptor antagonist	Traxoprodil (CP-101,606)	II	Adjunct	12	Low, high-dose infusion	Single dose	Change in Dyskinesia Rating Scale score	Positive (dose-related side effects)	[71]
	Memantine	II	Adjunct	12	30 mg/day	2 week treatment	Change in dyskinesia score after single levodopa challenge	Negative	[72]
AMPA receptor antagonist	Perampanel	III	Adjunct	763	2 or 4 mg/day	30 weeks	Reduction in off time and severity of dyskinesias (UPDRS IV)	Negative	[82]
			Adjunct	751	2 or 4 mg/day	20 weeks	Reduction in off time and severity of dyskinesias (UPDRS IV)	Negative	[82]
mGluR5 negative allosteric modulator	Mavoglurant (AFQ056)	II	Adjunct	31	50–300 mg/day	16 days	Change in LFADLDS	Positive	[95]
			Adjunct	28	50–300 mg/day	16 days	Change in mAIMS score	Positive	[95]
			Adjunct	197	20, 50, 100, 150, or 200 mg/day	13 weeks	Change in mAIMS score	Positive (for 200 mg dose)	[96]
	Dipraglurant (ADX48261)	II	Adjunct	76	Dose titration to 300 mg/day	4 weeks	Change in mAIMS score	Positive (on Days 1, 14)	[99]

Table 1 (continued)

Mechanism	Drug	Phase	Use	n	Dose	Duration	Primary Outcome	Result	Ref
Noradrenaline									
α2 Adrenergic receptor antagonist	Fipamezole	II	Adjunct	179	90, 180, or 270 mg/day	4 weeks	Change in levodopa-induced dyskinesia scale	Negative	[109]
Serotonin									
5-HT_{1A} receptor agonist	Sarizotan	II	Adjunct	398	2, 4, or 10 mg/day	12 weeks	Change in diary-based *on* time without dyskinesias	Negative	[110]
GAIT AND BALANCE									
Cholinesterase inhibitor	Donepezil	IV	Adjunct	23	5–10 mg/day	6 weeks	Reduction in fall frequency	Positive	[119]
Noradrenergic reuptake inhibitor	Methylphenidate	IV	Adjunct (+ STN DBS)	69	1 mg/kg/day	90 days	Change in number of steps in stand-walk-sit test	Positive	[125]
			Adjunct	23	Up to 80 mg/day	12 weeks; 3-week washout	Change in gait composite score	Negative	[126]
NMDA receptor antagonist	Memantine	IV	Adjunct	25	20 mg/day	90 days	Change in stride length	Negative	[127]

GABA = gamma-aminobutyric acid; GAD = glutamic acid decarboxylase; 5-HT = serotonin; NMDA = *N*-methyl-D-aspartate; AMPA = alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid; mGlu = metabotropic glutamate; AAV = adeno-associated virus; STN = subthalamic nucleus; DBS = deep brain stimulation; UPDRS = Unified Parkinson Disease Rating Scale; LFADLDS = Lang-Fahn Activities of Daily Living Dyskinesia Scale; mAIMS, modified Abnormal Involuntary Movement Scale

to convert STN neurons from being excitatory to inhibitory [40]. In an initial phase I, open-label study, 12 patients with advanced PD were followed for 12 months after unilateral injection of AAV–GAD into the STN. Improvements in contralateral on and off motor function were observed 3 months after the injection and persisted for 12 months [41]. In a phase II double-blind, randomized trial comparing bilateral delivery of AAV2–GAD to sham surgery, patients receiving active gene therapy demonstrated a significant improvement in motor Unified Parkinson's Disease Rating Scale score in the off state, but not the on state, at 6 months [42]. Despite these initial promising proof-of-principle findings for non-dopaminergic modulation of STN neurotransmission and for gene therapy in PD, the long-term follow-up study has been terminated owing to financial reasons [43] and there are no plans for phase III studies.

Serotonin

In PD, serotonergic neurons in the raphe nuclei degenerate, leading to a reduction in 5-HT levels [7]. Loss of 5-HT is thought to contribute to both motor and non-motor symptoms. In preclinical models, several 5-HT_{1A} receptor agonists have shown efficacy in improving motor activity and reducing dyskinesias. However, interpretation of these results is complicated in that these agents can also interact with other receptors. In levodopa-treated parkinsonian rats, the partial 5-HT_{1A} receptor agonist piclozotan improved motor function [44]. A randomized pilot study using piclozotan in a small number of PD patients on levodopa was also reported to show improvements in off and on time without dyskinesias [45, 46]. However, results have been published only in abstract form and additional trials have not been registered.

Pardoprunox (SLV308) is a full 5-HT_{1A} agonist that also has partial dopamine D2/D3 agonist properties. As monotherapy in animal models it reduced parkinsonian symptoms and induced only mild dyskinesias [47, 48]. In a double-blind study of pardoprunox in early PD, treatment resulted in improvement in motor function and activities of daily living [49]. Two large, randomized, phase III dose-finding trials also showed significant improvement in motor function, although dropout rates were high owing to treatment-emergent adverse events (e.g., nausea, somnolence, and dizziness) at higher doses [50]. As adjunctive therapy to levodopa, pardoprunox reduced off time and improved on time without troublesome dyskinesias in a phase III study [51]. However, a high dropout rate was similarly noted with the selected dose range, and the most recent registered clinical trial of pardoprunox was terminated "due to strategic considerations" [52].

Levodopa-induced Dyskinesias

Repeated administration of levodopa results in the development of motor complications, including involuntary dyskinesias. Age of PD onset, disease severity, and high levodopa dose are risk factors for the development of levodopa-induced dyskinesias (LID). Based on a literature review, the rate of development of dyskinesias has been reported to be about 35–40 % by 4–6 years of treatment, and nearly 90 % within a decade [53]. The mean time to onset of dyskinesias in a recent community-based study was 6.6 years [54]. LID can be clinically expressed in a variety of ways, occurring when levodopa effects are maximal (peak-dose dyskinesias, generally choreiform, but may be dystonic), with rising or falling levels of medication (diphasic dyskinesias), or at low levels of levodopa (off-period dystonia) [55, 56].

Evidence from postmortem and pharmacological preclinical studies supports a role for multiple non-dopaminergic systems in the development of LID [57–59]. These studies have led to exploratory trials investigating drugs targeting other neurotransmitters as adjunctive therapy with the goal of decreasing LID without compromising motor function.

Glutamate

Glutamate is the most abundant excitatory neurotransmitter in the brain and is directly involved in activating basal ganglia motor circuits. Loss of nigrostriatal dopamine input is believed to induce changes in synaptic connectivity in the striatum [60]. Repeated exposure to dopaminergic drugs, particularly in a hypodopaminergic parkinsonian state, results in maladaptive plasticity in glutamatergic synapses that contributes to the expression of dyskinesias [57, 61, 62].

Glutamate signaling in the CNS is mediated by a variety of receptors, including ionotropic receptors (those that directly conduct ion flow in response to glutamate binding) and metabotropic receptors (those whose actions are mediated via intracellular signaling pathways). Among ionotropic receptors, *N*-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA) receptors have been most extensively studied for a possible role in LID [59, 63].

Changes in NMDA receptor levels, phosphorylation state, and cellular distribution have been identified in the dyskinetic state in animal models [59]. Highlighting the complexity of antidyskinetic strategies targeting these receptors, preclinical studies using NMDA antagonists directed at specific receptor subunits in nonhuman primate models have yielded conflicting results. In one study, a negative allosteric modulator (Co-101,244/PD-174,494) acting on NR2B receptors decreased LID, while antagonists with increased specificity for NR1A/NR2A receptors exacerbated dyskinesias [64]. In contrast, another NR2B-specific antagonist (traxoprodil, CP-101,606) increased severity of dyskinesias in levodopa-treated animals [65].

As clinical support for a role for NMDA receptor modulation as an approach to treat LID, the nonselective NMDA antagonist amantadine is currently the only accepted treatment for dyskinesia in PD [66]. It has been recommended by the American Academy of Neurology for the treatment of dyskinesias (level C evidence) [67], and has also been suggested to be efficacious in treatment of LID in an evidence-based review by the Movement Disorders Society [68]. In a recent double-blind, randomized, placebo-controlled cross-over study of 36 patients with PD and dyskinesias, 64 % of patients showed improvement in LID [69]. Treatment with amantadine can also be limited by neuropsychiatric and other side effects. Remacemide, another nonselective NMDA antagonist, did not show a significant benefit in reducing dyskinesias in a randomized, controlled trial [70]. Hence, there is a need to develop better NMDA receptor antagonists with antidyskinetic properties.

To date, several small pilot studies have been conducted investigating other NMDA receptor blockers in PD. Traxoprodil, an antagonist selective for NR2B subunits, reduced the maximum severity of acute LID by approximately 30 % in response to a 2-h levodopa infusion in 12 PD patients with motor fluctuations and dyskinesias, but did not improve parkinsonism and caused dose-related neuropsychiatric side effects [71]. However, memantine, an NMDA receptor antagonist approved for treatment of dementia, did not improve dyskinesias in a small cross-over study [72], although there are case studies reporting a positive response [73–75]. An early small, double-blind, cross-over study suggested that the NMDA antagonist dextromethorphan may be effective in reducing LID [76]. AVP-923, a combination agent combining dextromethorphan and quinidine that has been approved for treatment of pseudobulbar affect [77], is currently being studied to assess its efficacy in reducing dyskinesias in a small phase IIa study [78]. Interestingly, recent preclinical data suggests that the potential antidyskinetic effect may be mediated by indirect 5-HT_{1A} agonism rather than through an NMDA antagonist effect [79].

The role of AMPA receptors in the development of LID has received less attention, although the AMPA antagonists talampanel (LY-300,164) [80] and topiramate [81] reduced LID in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned primates. To date, 2 phase III human clinical trials using the AMPA antagonist perampanel as potential treatment for PD motor fluctuations have been published, but the drug did not show benefit either in reducing dyskinesias or "off" time [82]. Two small phase II studies investigating talampanel as an antidyskinetic agent have been completed, but results have not been published [83, 84].

Currently, metabotropic glutamate (mGlu) receptors are receiving significant attention as potential therapeutic targets [85, 86]. In particular, mGlu5 receptors are highly expressed

in the striatum and globus pallidus. Expression of mGlu5 is upregulated in MPTP-lesioned primates treated with levodopa, and this increase is associated with the development of LID [87]. Administration of mGlu5 antagonists has been shown to attenuate abnormal involuntary movements in rodent models [88, 89] and LIDs in primates [90–93].

Negative allosteric modulators (NAM) of G-protein-coupled receptors target binding sites distinct from the active site and inhibit the response to endogenous ligand. Drugs targeting allosteric sites may provide greater receptor selectivity and potentially decrease adverse side effects [94]. Clinically, the selective mGlu5 NAM mavoglurant (AFQ056) was demonstrated to show a significant antidyskinetic effect in 2 small phase II randomized clinical trials [95]. Findings from a larger dose-finding study of 197 patients with PD and dyskinesias provided further evidence of anti-dyskinetic benefit without worsening of parkinsonism [96]. A phase II study exploring the efficacy and safety of a modified release form was also recently completed [97]. Another mGlu5 NAM, dipraglurant (ADX48261), has similarly been under investigation as a putative antidyskinetic agent [98]. Although not yet published, preliminary results presented in abstract form suggest a significant reduction in peak dose LIDs without affecting levodopa efficacy [99]. Together, these results suggest that mGlu5 antagonists offer promise for the treatment of LID.

Noradrenaline

Noradrenaline exerts its action by binding to G-protein-coupled adrenergic receptors, which are expressed in the striatum, STN, and substantia nigra [100]. Of particular interest are α2 adrenergic receptors, which may act to modulate GABA [101, 102] and dopamine release [103]. In pharmacological studies using primate models, α2 antagonists have been shown to reduce LID [104, 105], possibly through preferential effects on the direct pathway [57]. These preclinical findings have motivated clinical trials exploring these agents as potential antidyskinetic therapies.

Pilot studies using idazoxan yielded conflicting results [106, 107], and this drug is no longer in clinical development for PD. Currently, the selective $\alpha_{2A/2C}$ receptor antagonist fipamezole is being studied for LID. An initial small pilot study demonstrated good tolerability and suppression of LID without exacerbating parkinsonian symptoms [108]. In a phase II study conducted in the USA and India, fipamezole failed to show a statistically significant reduction in dyskinesias [109]. However, separate outcome analysis of the US patients did show a benefit at the highest dose used; it has been proposed that this differential result may be owing to heterogeneity between the US and Indian study populations. An additional clinical trial may be helpful to determine whether fipamezole is indeed useful for treatment of LID.

Serotonin

Serotonin has also been implicated to play a role in LID, and 5-HT_{1A} receptor agonists and 5-HT_{2A} receptor antagonists have been explored as promising antidyskinetic agents. In a large phase IIb study, sarizotan, a full 5-HT_{1A} agonist with additional affinity for dopamine D3/D4 receptors, did not show benefit in increasing on time without dyskinesias, and resulted in increased off time at higher doses [110]. Eltoprazine, a mixed 5-$HT_{1A/1B}$ receptor agonist, is effective in suppressing LID in animal models [111]. A small, human, randomized clinical trial has been completed in Sweden, but results have not yet been published [112]. It has been proposed that drugs aimed at reducing LID by modulating serotonergic function may need to demonstrate anatomic selectivity, as well as receptor selectivity [113].

Gait and Balance

Postural instability and gait difficulty are cardinal features of idiopathic PD, but typically do not cause prominent functional disability until later stages of disease. In particular, patients at more advanced stages of PD may become unable to initiate locomotion and develop freezing of gait (FOG) [114]. FOG is often associated with gait imbalance and can result in falls [115]. In some cases, FOG may respond to dopaminergic therapy at earlier stages [116]; however, PD-associated gait disorders may become progressively resistant to dopamine replacement or can be unresponsive from the start. Neurodegeneration in non-dopaminergic brainstem structures may contribute directly to this lack of response [117]. Cholinergic neurons in the pedunculopontine tegmental nucleus (PPN) and the prefrontal and frontal cortex are thought to be involved in gait control. Noradrenaline-producing cells in the locus coeruleus are also severely affected in PD. As a result of striatal dopamine depletion, excessive glutamatergic activity at projections from STN to PPN may also contribute to locomotor dysfunction. Interest in the role of PPN in PD gait disorders has been supported by the finding that low-frequency deep brain stimulation may reduce falls and FOG, either alone or in combination with high-frequency STN stimulation [118].

Strategies to increase ACh transmission have been used to target gait and balance symptoms unrelated to FOG. A small, randomized, placebo-controlled, crossover study in PD patients with falls showed that the centrally-acting cholinesterase inhibitor donepezil reduced falls by approximately half [119]. A single-center study in the UK is similarly exploring the effects of rivastigmine on gait and balance [120]. In the striatum, nicotinic ACh receptors are located presynaptically, and include subtypes α4β2, α6β2, and α7 receptors. A single-site study investigating the use of varenicline, a partial

α4β2 and full α7 agonist used as an aid for smoking cessation, to improve balance is ongoing [121].

Methylphenidate is an amphetamine-like stimulant that inhibits presynaptic noradrenaline and dopamine transporters. Three small pilot studies using different dosing protocols demonstrated improvement in various gait measures, including gait speed and freezing [122–124]. Two subsequent randomized studies have been completed and reported conflicting results. In a study of 69 PD patients treated with STN–DBS, methylphenidate treatment improved the number of steps in the stand-walk-sit test; the treated group experienced significantly more adverse events [125]. However, another trial of 23 patients did not show any improvement in a gait composite score of stride length and velocity [126].

A recent study of 25 patients explored the use of the NMDA receptor antagonist memantine as treatment for axial symptoms of PD. Although the treated group showed improvement in axial motor symptoms and dyskinesias, no improvement was noted in stride length [127]. Similarly, a randomized, double-blind, placebo-controlled crossover trial of the non-specific NMDA antagonist amantadine failed to show benefit against FOG resistant to dopaminergic therapy [128].

Conclusions

Dopamine deficiency due to degeneration of the nigrostriatal pathway is the primary cause of motor symptoms in PD. Nevertheless, multiple other neurotransmitter systems play an important role in modulating basal ganglia function and motor control. Targeting these systems, in particular, offers potential approaches to treating motor complications of dopamine replacement and symptoms that are resistant to dopaminergic therapy. At first glance, candidate non-dopaminergic agents would appear to be the proverbial "low-hanging" fruit in the PD pipeline. Receptors for neurotransmitters, including adenosine, GABA, serotonin, glutamate, and noradrenaline, have been well characterized biochemically with extensive knowledge of their neuroanatomic distribution and intracellular signaling pathways. Moreover, preclinical studies in rodent and nonhuman primate models have demonstrated effectiveness in reducing parkinsonian symptoms.

Based on the promise of the animal studies and early phase clinical studies, a number of randomized clinical trials directed at a variety of neurotransmitter targets have been completed [129]. Unfortunately, no compound specifically targeting non-dopaminergic pathways has yet received broad regulatory approval of an indication for use in the therapeutic armamentarium for PD. Drawing on previous studies, a potential hurdle may be finding agents that show high receptor specificity and also target specific brain regions. For example, the presence of multiple glutamate and serotonin receptor subtypes offers the potential for designing drugs that act on one, or a narrow, subset of receptors. However, the widespread distribution of these receptors throughout the CNS poses the challenge of finding doses that do not cause limiting side effects through action at undesired neuroanatomic sites.

Adenosine A_{2A} receptors have been an attractive potential target, as they are highly enriched in the striatum. Phase III studies investigating 2 A_{2A} antagonists, istradefylline and preladenant, have been conducted, and while phase II studies have consistently shown benefit in reducing motor fluctuations, the large phase III studies have yielded conflicting results, slowing progression through the therapeutic pipeline. These discrepancies raise questions about the design of clinical trials addressing motor fluctuations. Determination of on/off fluctuations relies on patient diaries, which may be subject to variability despite appropriate training. Also, while there are multiple dyskinesia rating scales [130], the clinical variability in the types of dyskinesias and their timing offers challenges in quantifying response to treatment. There is similarly a need to define the most appropriate outcome measures for trials focusing on PD gait symptoms. The recent approval of istradefylline in Japan [26] will hopefully provide additional experience about the effect of A_{2A} antagonists as adjunctive therapy. Additional phase III studies with mGlu5 receptor antagonists will be necessary to confirm the initial promising results from phase I/II studies.

Given the complexity of the pharmacology of dopamine-induced and dopamine-refractory PD symptoms, it may be necessary to target multiple non-dopaminergic systems in order to optimize clinical response while minimizing side effects from any particular pathway. This presents obvious obstacles to clinical trial design. However, despite the challenges thus far, ongoing development of these strategies remains a critical and hopeful pursuit toward improved treatment of PD.

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Appendix D

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Appendix D

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Appendix E

Postmortem Brain Levels of Urate and Precursors in Parkinson disease and Related Disorders

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Abstract

Background—Increasing evidence suggests that urate may play an important role in neurodegenerative disease. In Parkinson disease (PD) higher, but still normal, levels of blood and CSF urate have been associated with a lower rate of disease progression.

Objective—We explored the hypothesis that lower levels of urate and its purine precursors in brain may be associated with PD and related neurodegenerative disorders, including Alzheimer's disease (AD) and Lewy body dementia (DLB).

Methods—Human postmortem brain tissues were obtained from PD, AD, and DLB patients and non-neurodegenerative disease controls. We measured urate and other purine pathway analytes in frontal and temporal cortex, striatum, and cerebellum, using HPLC with electrochemical and ultraviolet detection.

Results—Age was well-matched among groups. Mean postmortem interval (PMI) for samples was 16.3 ±9.9 hrs. Urate levels in cortical and striatal tissue trended lower in PD and AD compared to control in males only. These findings correlated with increased urate in male vs. female control tissues. By contrast, in DLB urate levels were significantly elevated relative to PD and AD. Measurement of urate precursors suggested a decrease in xanthine in PD compared to AD in females only, and relative increases in inosine and adenosine in DLB and AD samples among males. Xanthine and hypoxanthine were more concentrated in striatal tissue than in other brain regions.

Conclusions—Though limited in sample size, these findings lend support to the inverse association between urate levels and PD, as well as possibly AD. The finding of increased urate in DLB brain tissue is novel and warrants further study.

Keywords

Uric acid; purines; neurodegeneration; parkinsonism; Lewy body disease; Alzheimer's disease

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Introduction

Increasing clinical, epidemiological, and laboratory evidence suggests that urate (or uric acid) may play a role in neurodegenerative disease, and Parkinson disease (PD) in particular. Urate is a natural antioxidant, found abundantly in blood and human brain tissue due to mutations of the *urate oxidase* (*UOx*) gene during primate evolution.[1] In humans, urate is thus the enzymatic end product of purine metabolism (Figure 1) and circulates at high plasma concentrations. Urate's antioxidant capacity is comparable to that of ascorbate[2, 3] and suggests that the loss of urate oxidase activity in our primate ancestors may have provided additional antioxidant health benefits.[4] Urate also inhibits free-radical formation and forms complexes with iron (particularly Fe^{3+}), crucial to limiting oxidative damage.[2] As oxidative stress is thought to contribute to loss of nigrostriatal dopamine neurons in PD and the pathophysiology of other neurodegenerative disorders, levels of urate and its metabolites may help determine disease susceptibility and predict rate of progression.[5, 6]

In the early 1990s Church and Ward provided initial, post-mortem evidence that nigrostriatal levels of urate (but not ascorbate) as well as dopamine are reduced in PD.[7] Epidemiological and clinical studies subsequently linked lower urate levels to a greater risk of PD and a faster rate of its progression.[8–10] Lower urate levels have likewise been reported for patients with mild cognitive impairment and Alzheimer disease (AD).[11–13] Similarly, higher urate has been associated with reduced progression in Huntington's disease[14] and multiple system atrophy.[15] Furthermore, recent data suggest a potential link between higher plasma urate levels and reduced progression of cognitive decline and adjusted risk of dementia.[16, 17] However, other studies have linked higher urate to an increased risk of dementia though these generally have not been adjusted for cerebral ischemia, which is frequently comorbid with both dementia and elevated urate levels and may mediate the association between urate and cognitive dysfunction.[18]

In this study we explored the hypothesis that lower brain urate levels may be associated with PD and related neurodegenerative disorders, including AD and dementia with Lewy bodies (DLB). A secondary aim was to determine whether levels of urate and its precursors vary among brain regions and correlate with affected areas in each disease. We analyzed human postmortem brain tissue obtained from the MassGeneral Alzheimer Disease Reserch Center (ADRC)/Harvard NeuroDiscovery Center neuropathology core from PD, AD, and DLB patients and age-matched non-neurodegenerative disease controls. Urate pathway analytes were measured in multiple brain regions, including frontal and temporal cortex, striatum, and cerebellum using HPLC with electrochemical and ultraviolet (UV) detection.

Methods

Standard protocol approvals and patient consents

Postmortem tissue collection and protocols were approved by the Partners/Massachusetts General Hospital Institutional Review Board. Prior written, informed consent was obtained from brain donor participants or from their family members or authorized representatives.

Tissue selection

Brain samples were obtained from the MassGeneral ADRC/Harvard NeuroDiscovery Center neuropathology core B repository based on tissue availability and confirmed neuropathological diagnosis. Relevant clinical information such as age, gender, race, comorbidities, and clinical diagnosis was acquired from the brain bank database. Criteria used for neuropathological diagnosis included those established by the London Brain Bank for PD,[19, 20] the DLB Consortium,[21] and the National Institute on Aging and Reagan Institute Working group for AD.[22] Fresh frozen tissue (~100–200 mg, stored at −80 °C)

from PD, DLB, AD and age-matched, non-neurodegenerative disease control brains were collected and included samples from frontal and temporal cortices, striatum (caudate and putamen), and cerebellum. There was insufficient midbrain tissue for adequate sampling. Cases with combined AD and Lewy body pathology, and those with extensive cerebrovascular disease considered likely to cause dementia, were excluded. To help ensure stability of urate and metabolites, the average postmortem interval (PMI) for cases was kept as short as possible (generally <24hrs) and matched across the disease and control groups (Table 1). 5–10 samples for each disease state, gender, and specified brain region were examined.

Urate and precursor measurements

We compared urate pathway metabolites in human postmortem tissue across select brain regions and neurodegenerative diseases, including PD, DLB, AD, and controls. Tissue samples were homogenized on ice in 50 mM phosphoric acid solution (containing 0.1 mM EDTA) with 50 μM methyldopa and 1 μM 3,4-dihydroxybenzylamine as internal standards, and then centrifuged at 16K × g for 15 min. The supernatant was filtered through a 0.22 μm Spin-X (Costar) cellulose acetate filter.[23] Purines in the brain homogenate filtrates were separated over a reverse-phase HPLC column, and then measured in the effluent by serial UV and electrochemical detectors. Specifically, adenosine, inosine and hypoxanthine were quantified based on UV absorbance at 254 nm whereas urate and xanthine were quantified based on oxidation at a coulometric detector set at 150 mV and 450 mV, respectively.[24] For calibration standard concentrations of each purine were also measured. All data were collected using a CoulArray Data Station with 3.0 software (ESA Biosciences, Chelmsford, MA) with autorange gain enabled. Measurements for each sample were normalized to the methyldopa standard peak and wet weight of tissue analyzed (expressed as ng of analyte per wet weight of brain tissue (wwt) in figures).

Statistical analysis

All statistical analyses were performed in SPSS 20.0 (IBM Corp.). For comparison of group statistics, we performed analysis of variance (Kruskal-Wallis, alpha = 0.05) with post-hoc Dunn's multiple comparison tests. Linear regression analysis was done on urate and precursor measurements for all areas (average) versus PMI and age. Normalized data for urate and precursors were analyzed by multivariate GLM (MANCOVA) with disease, brain region, and gender as independent variables and with age and PMI as covariates. Posthoc pairwise comparisons were performed with Bonferonni correction. Data in graphs are expressed as mean ±SEM.

Results

A total of 62 cases was collected and included 17 PD, 13 DLB, 19 AD, and 13 age-matched non-neurodegenerative disease controls. Controls died of various causes, including cardiovascular disease, pneumonia, cancer, and gastrointestinal bleed.. For each group basic demographic and specimen features are displayed in Table 1. Disease and control groups were generally well-matched for age and PMI. There was no significant difference in age among groups with mean age for all groups being 79.2 years (SD ±9.0). Overall mean PMI was 16.1 hours (SD ±10.4) and differed among groups ($H = 8.6$, $df = 3$, $p = 0.035$) with PMI for AD (12 hours ±7.5) being significantly lower than for controls (22.7 hours ±12.4). Given uncertainty over the stability of urate and enzymatic precursors in postmortem tissue, we performed regression analysis of brain urate and precursor levels versus PMI and age (Table 2). Although we found no significant correlation with urate levels (Figure 2A), PMI appeared to predict higher levels of xanthine. There was also a trend for higher levels of hypoxanthine, whereas lower levels of both inosine and adenosine were suggested. The

cause of these trends is unclear, but may indicate postmortem, active enzymatic or non-enzymatic metabolism of purines early in the pathway. Regression analysis revealed a non-significant trend toward increased urate levels with age but is consistent with that found in serum urate levels (Figure 2B).[25, 26] Among precursors only xanthine showed a significant correlation and decline in levels with age.

Urate and Precursor Levels in Parkinson Disease and Gender Differences

Two-way analysis of primary measurements of urate in postmortem brain samples was restricted to disease relevant regions, including cortical and striatal samples only, and showed main effects for both disease (F[3,136]=7.44, p<0.0005) and gender (F[1,138]=6.38, p=0.013), as well as a significant interaction between disease and gender (F[3,136])=4.10, p=0.008). In control tissue urate levels in males were significantly higher (8.21 ±1.0 ng/mg wwt, p=0.014) than in females (4.06 ±1.44), consistent with that reported in serum.[27, 28] Urate levels in PD trended lower than control (p=0.096) among males, whereas there was no difference among females (Figure 3). Interestingly, urate in DLB among males was significantly elevated compared to PD (p<0.0005) and AD (p<0.0005). Analysis of xanthine levels also showed main effects for disease (F[3,136]=3.53, p=0.017) but not gender, though there was a significant interaction between disease and gender (F[3,136]=7.75, p<0.0005). Pairwise comparisons showed that xanthine levels in PD were significantly lower (p=0.019) in females (22.6 ±3.7) than in males (32.9 ±2.3), whereas in control and DLB levels were higher in females (42.0 ±4.1, p=0.002 and 43.1 ±2.9, p<0.000; respectively). Additionally, among females xanthine was reduced in PD compared to DLB (p<0.0005) and to control (p=0.004). No significant differences in xanthine levels were found in males. There were no interactions or main effects detected for hypoxanthine levels either. However, interaction between disease and gender effects on inosine levels trended toward significance (F[3,136]=2.21, p=0.09). Post-hoc analysis revealed that in PD, females have significantly elevated brain levels of inosine (p=0.012) compared to males (149.6 ±20.1 vs 89.1 ±12.6, respectively). Among males, inosine levels in AD were also increased (137.8 ±10.8, p=0.025) relative to PD. For adenosine, there were no significant main effects due to the high variability of adenosine readouts, although pairwise comparisons suggested differences in levels for DLB between male and female (p=0.01) tissues, as well as between male DLB and control (p=0.005) and PD levels (p=0.006).

Urate/precursor levels by region and disease

Analyses of urate and precursor levels in postmortem brain samples by region and disease include both genders pooled (Figure 4). We also performed separate male and female analyses which showed similar results, though there were limited female PD samples for all brain regions (data not shown). For urate, there were main effects for region (F[3,162]=3.09, p=0.029) and disease (F[3,162] = 8 in striatum.83, p < 0.0005; Figure 4A, B) on postmortem brain levels. Simple effects analysis indicated that cerebellar urate levels were higher than that in striatum (p=0.041). Among disease groups, urate was elevated in DLB brains vs. control, AD, and PD (p = 0.029, <0.0005, and <0.0005, respectively). Posthoc analyses similarly showed significant increase in urate in cerebellum for DLB compared to AD (p=0.004). Though urate in PD and AD appeared lower than control, differences did not reach significance. By contrast, brain xanthine levels did not differ among disease groups but trended higher in the striatum compared to other tissues (F[3,162] = 9.15, p < 0.0005). Likewise, there was no association between hypoxanthine levels and disease, but significant regional differences in hypoxanthine (F[3,162] = 11.66, p < 0.0005) were noted for striatum compared to frontal and temporal cortices (p<0.0005), as well as cerebellum compared to frontal (p<0.0005) and temporal (p=0.024) cortices. Inosine levels displayed a disease effect (F[4,162] = 2.71, p=0.047) with relative increase in AD vs. control (p = 0.20), but no significant regional associations. Although adenosine values were low and variable, we

detected a main effect for disease (F[3,162]=5.32, p=0.002) with DLB levels being higher than those in PD (p=0.015) and control (p=0.006).

Discussion

Urate, the end product of enzymatic purine metabolism in humans, has emerged as a potential biomarker for PD with serum and CSF levels correlating inversely with risk and progression rates.[9, 28] Recent studies also indicate a potential link between urate, cognitive decline, and AD.[11, 12, 16, 17] In this study we analyzed postmortem brain levels of urate and its purine precursors among multiple select brain regions and neurodegenerative diseases, including PD, DLB, and AD, to test the hypothesis that lower levels correlate with disease. In males, but not females, there was a clear trend toward lower urate levels in PD versus control brains, though levels did not quite reach significance (p=0.096). This finding correlated with significantly higher urate levels in control tissue in males vs. females, mirroring similar gender differences reported in serum.[29] Our findings are generally in agreement with Church and Ward (1994), who also found lower levels of urate in PD substantia nigra and striatum compared to control.[7] These findings appear to support the notion that in males PD brains may, as a result of lower urate levels, have a decreased antioxidant capacity and greater risk for oxidative damage and dopaminergic cells loss,[2, 5] consistent with epidemiological data suggesting an inverse correlation with risk and disease progression rate.[9, 10]

Conversely, a lower brain urate concentration in PD patients may be secondary to their putatively higher levels of oxidative stress and reactive oxygen species (ROS).[5] Urate is consumed as it exerts its antioxidant action, which entails the non-enzymatic oxidation of urate by ROS resulting in irreversible conversion to allantoin. Thus it would be informative to determine whether lower levels of urate in PD are associated with higher levels of allantoin, with a higher allantoin:urate ratio potentially serving as an index of oxidative stress[30] and potentially a more robust prognostic biomarker of PD compared to urate alone. Because urate's purine ring structure is disrupted upon its conversion to allantoin, the electrochemical or UV methods employed here were not adequate on their own to measure the analyte in brain tissue, but may be coupled with an allantoin derivitization step toward this end in future studies.

Whereas brain urate levels trended lower for PD, unexpectedly we found that urate appeared significantly elevated in DLB brains in all brain regions examined. Ours is the first study to our knowledge to demonstrate this finding in postmortem brain. Similar to PD, differences in urate in DLB reached significance only in males. Greater numbers are needed to increase the power of analysis however. Although dementia in some studies has been linked to higher urate levels, this association has been attributed at least in part to urate's covariance with vascular risk factors.[31–33] Indeed other studies have shown the opposite association,[11, 12] with *lower* urate levels linked to reduced risk of dementia after adjusting for potential cardiovascular confounds. Thus, the higher urate levels we observed in postmortem DLB brain could reflect associated vascular risk factors, which have been shown to be a determinant of DLB diagnosis.[34] Vascular confounds, however, would not readily explain why urate was elevated in DLB but not in AD, which is similarly thought to be more likely among those with vascular risk factors. Information on whether the DLB subjects differed from other groups based on vascular risk factors was not available in the present study, but would be helpful to factor into future studies investigating the role of urate in neurodegenerative diseases to which vascular disease may contribute.

The findings in DLB also contrast those of a recent study of urate levels in Lewy body disorders with or without dementia, which reported lower levels of CSF urate in dementing

Lewy body disorders (including DLB) compared to non-demented PD patients.[35] However, this study also reported differences between serum and CSF urate relationships to neurodegenerative diseases, suggesting that brain too may have a distinct association with urate. Thus the role of brain urate in DLB in particular remains unclear and warrants further study.

Gender differences seen in this study add to increasing data that the urate-PD link is stronger in men than in women. For instance, men with gout have decreased risk of PD whereas data for women were not significant, although interestingly use of anti-gout treatment was associated with reduced PD risk in both groups.[36] Serum levels also negatively correlated with risk of PD, disease duration, and daily levodopa dose in men, but again not in women, [37] though a trend toward reduced risk in women has been observed in at least one epidemiology study.[38] Studies on disease progression likewise show an inverse correlation between serum or CSF urate and rate of clinical decline that is significant in men but not in women.[10, 28] More recently, a similar inverse association between serum urate and presence of dopaminergic deficit on [I^{125}]β-CIT SPECT was seen in men but did not quite reach statistical significance (p=0.051) in women.[39] In our study lower brain urate levels in females correlated with those found in serum and CSF, and were not different among control and PD tissue, which may help to explain the lack of association in women. Precursor levels, however, differed only for inosine, which was significantly elevated in woman and not men, a finding not previously reported. Together these studies support the possibility that factors other than urate may play a more primary role for parkinsonism in women, and that perhaps their oxidative burden is not as high as in men, who require or produce more urate.

In addition to gender, we explored regional differences in urate and metabolite levels as differences might be expected to be more prominent in areas affected by disease, such as the nigrostriatal system in PD. Previous reporting of postmortem brain urate in PD also reported reduced urate concentration compared to that in control subjects but was based on a small sampling (n=4) and was limited to nigral and striatal tissues.[7] In combination with their results, the present findings suggest a more generalized reduction in urate in PD brain rather than one specific to the nigrostriatal system.

Among urate's immediate precursors, xanthine and hypoxanthine, we observed few disease-specific alterations with the exception of relative decrease in xanthine levels for PD compared to AD in females. Precursor levels, however, did appear to vary significantly by region in some disease as well as control brains, with striatal levels generally being the highest. Though consistent with a regionally specific decrease in striatal xanthine oxidase function there was no accompanying decrease in striatal urate, and the significance and reproducibility of these differences remain to be determined.

This study has several limitations including the small sample size, lack of relevant midbrain tissue, and limited corresponding clinical information. Tissue samples were primarily obtained from one source, the MGH-ADRC, and thus limited in scope and regions available. We further limited tissue samples, particularly in choosing controls, to those without concurrent severe cerebrovascular pathology and reported dementia that could confound urate measurements. Cardiovascular disease was reported in some cases (likely an underestimate given limited records) but other comorbidities, such as alcoholism, diabetes, and obesity, as well as medications were not detailed. Although cardiovascular risk is associated with elevated urate levels,[40] it remains unclear whether this translates to higher levels in brain.

In conclusion, this study provides further support for a role of urate in PD by strengthening the direct evidence that urate levels in degenerating brain tissue of male PD patients, as well as in their CSF and blood, are lower than in control subjects. Although we did not examine oxidative stress in brain tissues, previous studies suggest that oxidation of dopamine and other markers of neuronal integrity are increased in the PD nigrostriatal system and that urate may play an important antioxidant role.[7, 41] Testing in animal models of parkinsonism may help further clarify the role of urate. Based on findings herein, the importance of urate and its metabolites in other neurodegenerative disorders remains unclear and warrants future investigation.

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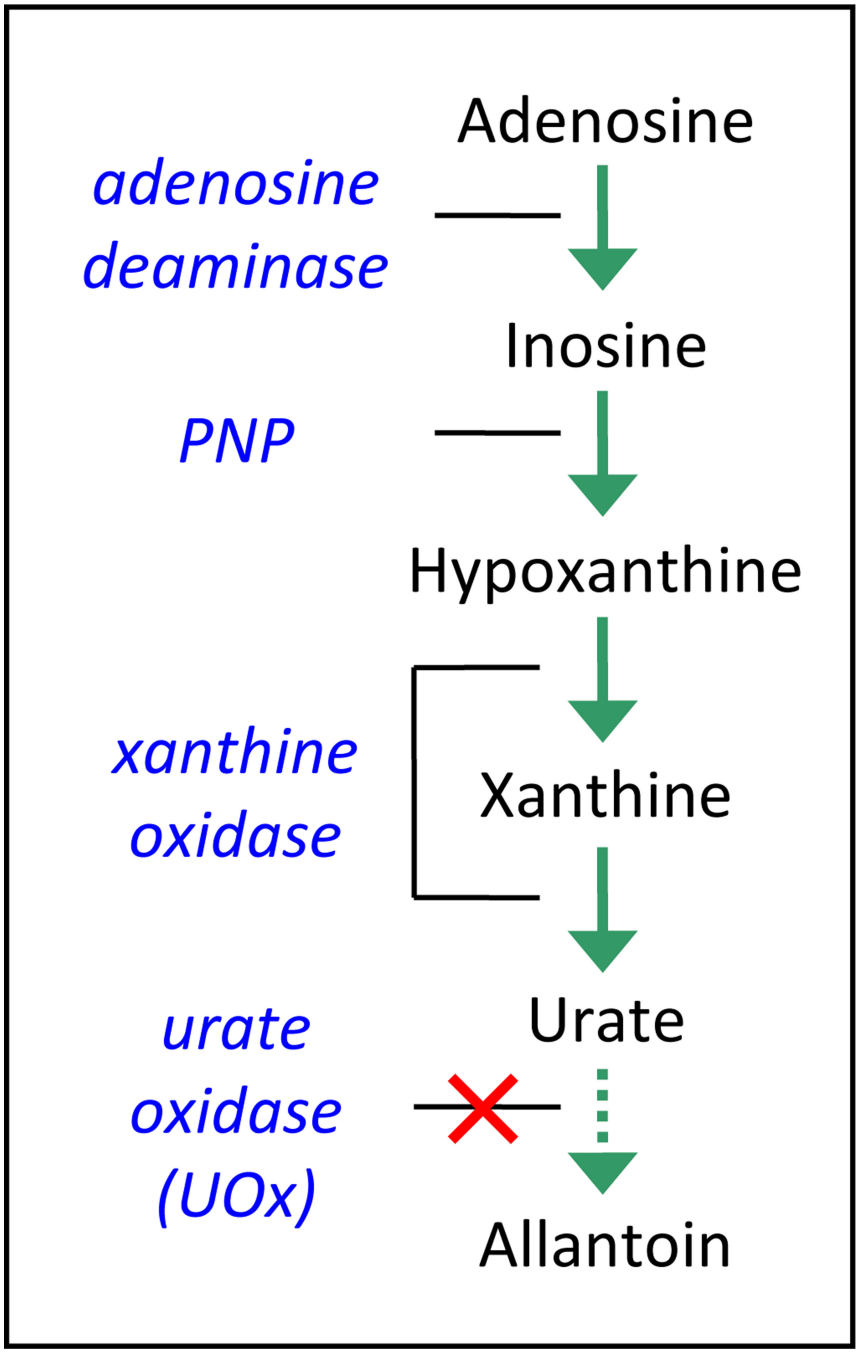

Figure 1. Metabolism of purines in humans

Loss of urate oxidase (UOx) function results in elevated urate. (PNP, purine nucleoside phosphorylase)

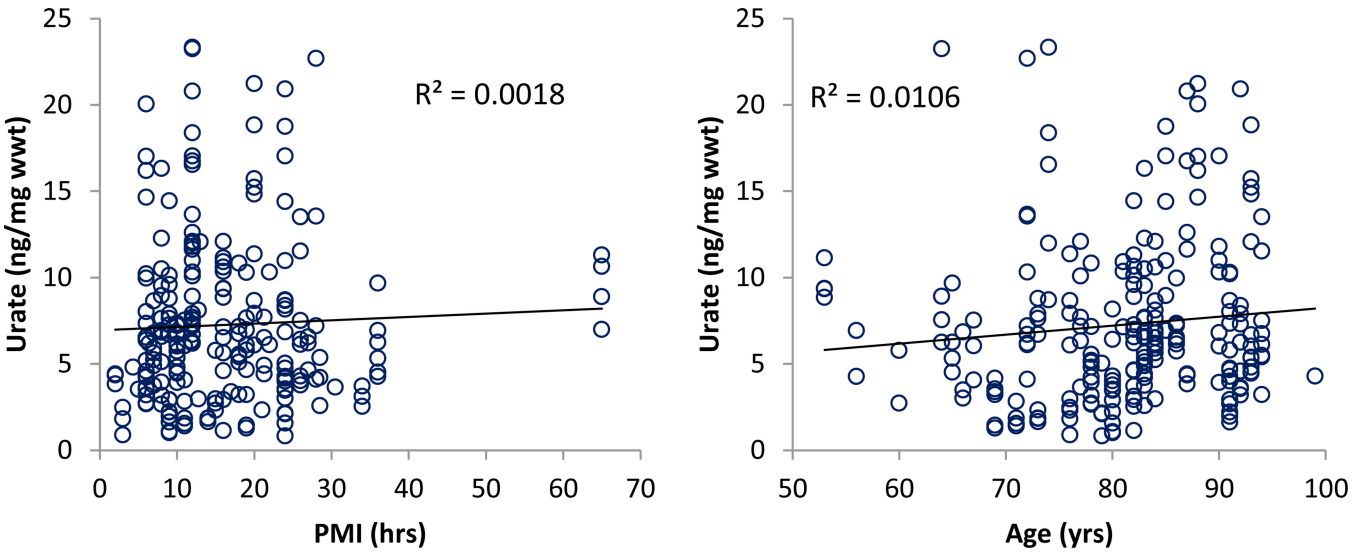

Figure 2. Regression analysis
of A) PMI vs. urate levels (β=0.042, p=.576), and B) age vs. urate (β=.114, p=.128). No significant relationships were found.

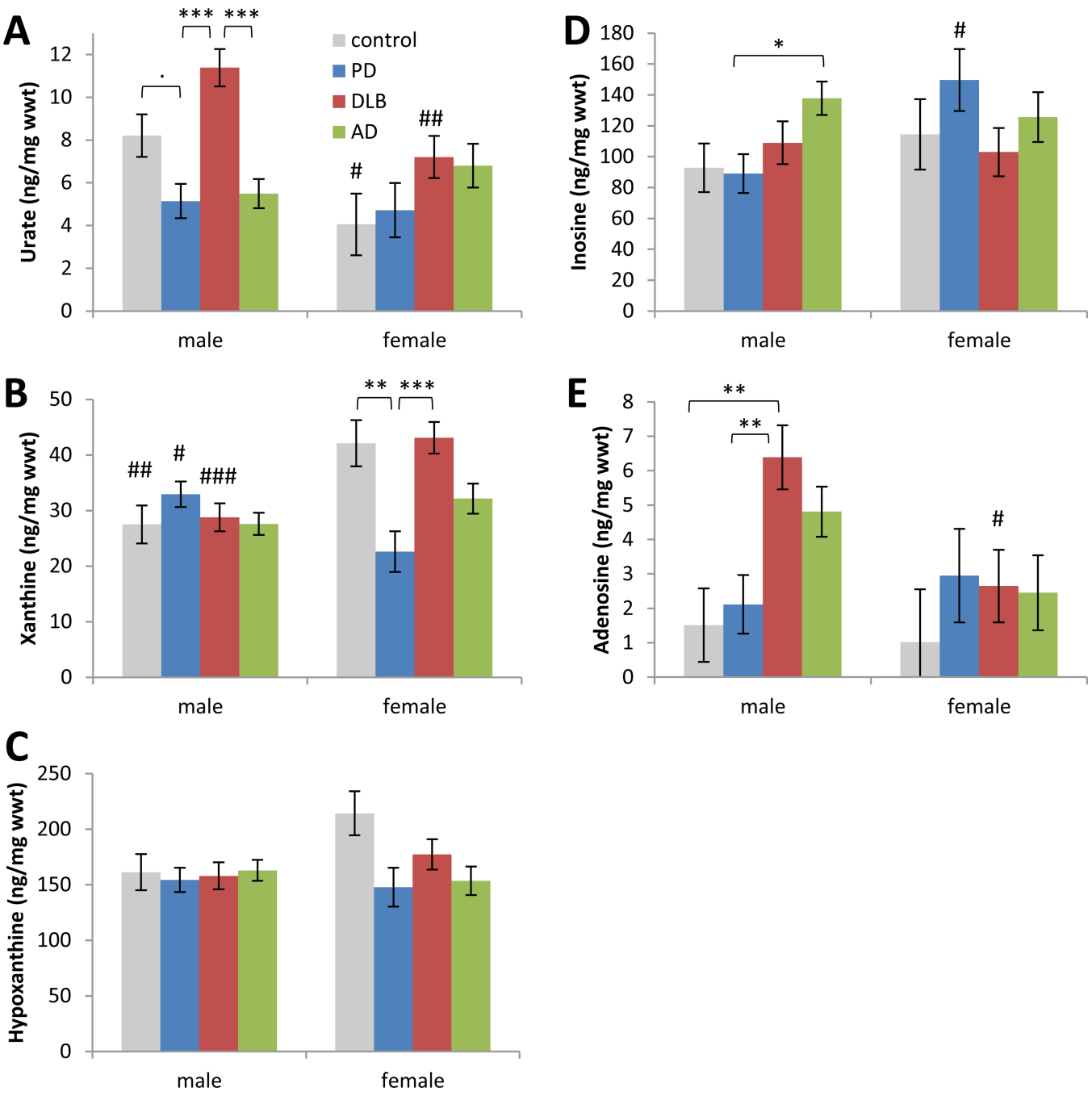

Figure 3. Disease versus gender differences in brain purine levels for non-neurodegenerative disease control, PD, DLB, and AD tissue

Mean levels are expressed as ng per wet weight tissue (mg). Urate levels (A) in controls are significantly lower in female vs male. Urate in PD males appears reduced and is more comparable to that in female control tissue, but represents a trend only, p=0.096. In contrast, urate in DLB is significantly elevated compared to PD and AD ($p < 0.0005$). Among precursors, xanthine (B) in PD is decreased in females vs males, whereas increased in DLB females. Xanthine in DLB females is also significantly elevated compared to PD. Inosine (D) levels are increased in AD compared to PD. In PD tissues, inosine is also significantly elevated in female vs male samples. Adenosine is increased in DLB relative to control and

PD among males. (· p<0.1, *p<0.05, **p<0.01, ***p<0.001) (gender comparison: #p<0.05, ##p<0.01)

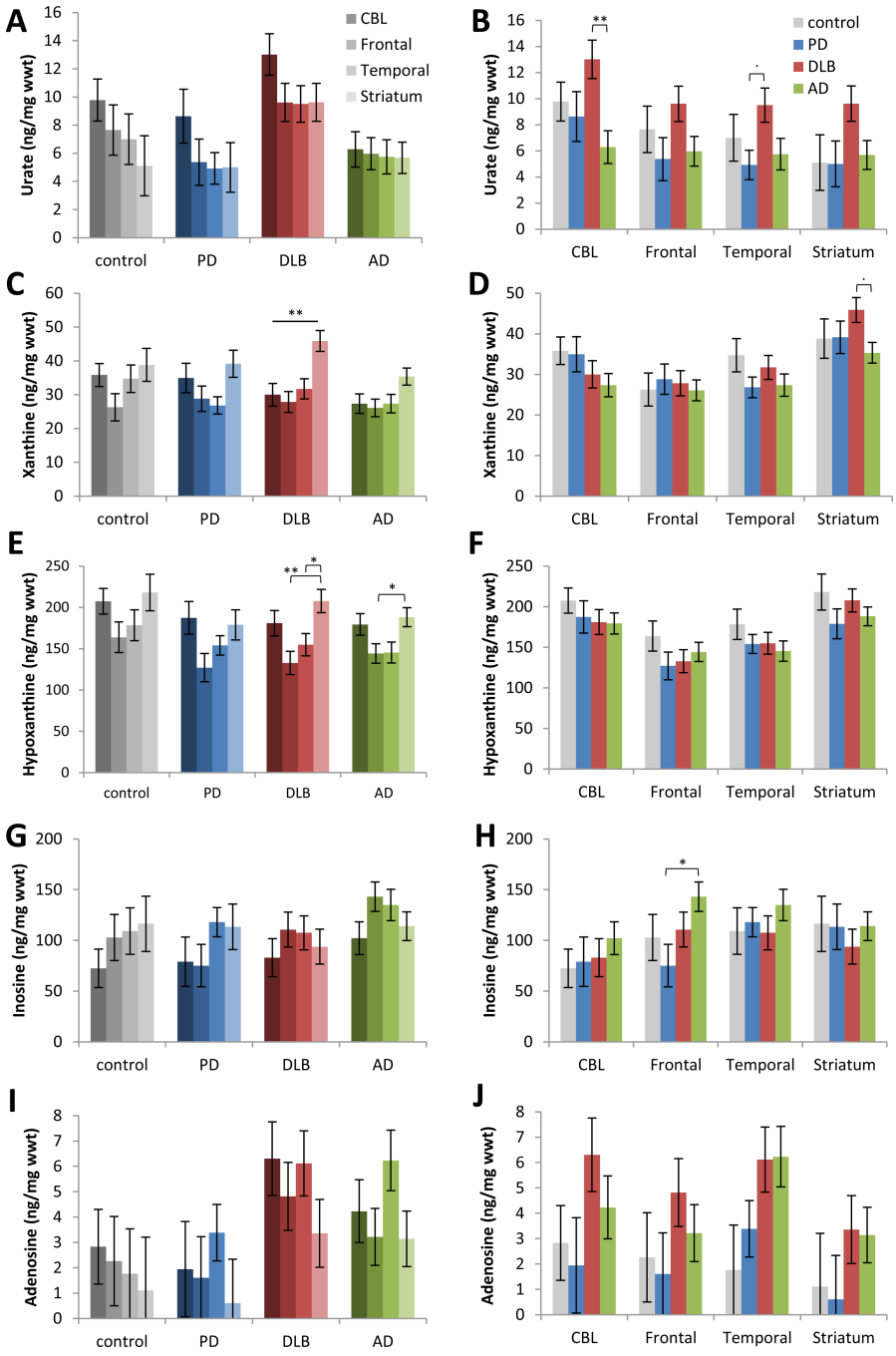

Figure 4. Region versus disease group differences in brain purine levels

Graphs of both disease vs. region (left) and region vs. disease (right) are shown for clarity. For urate (A,B) there is a clear trend toward lower urate in PD and AD, but significant elevation in DLB ($p < 0.0005$). Xanthine (C,D) shows no relation to disease, but is increased in striatum. Similarly, hypoxanthine (E,F) levels are greater in striatum and cerebellum (CBL) compared to frontal and temporal cortices ($p < 0.0005$). There is no disease effect. For inosine (G,H) no regional or disease effects are seen, though AD levels are greater than that in PD in frontal tissues. Adenosine (I,J) levels are increased in DLD compared to PD ($p=0.015$) and control ($p=0.006$). Significant interactions (post-hoc) between region and disease for urate and precursors are also shown (· $p<0.1$, *$p<0.05$, **$p<0.01$).

Table 1

Group Statistics

Values in same row for age and PMI not sharing the same subscript are significantly different ($p<.05$) for two-sided test of column means (Kruskal-Wallis test of disease versus age or PMI). PMI for control and AD are significantly different ($p=0.023$).

	Control	PD	DLB	AD	p
Total (n)	13	17	13	19	-
M/F	8/5	8/9	7/6	12/7	-
Mean Age in yrs (SD)	78.3 (11.3)$_a$	79.2 (7.5)$_a$	75.6 (9.8)$_a$	82.2 (7.6)$_a$	0.342
Mean PMI in hrs (SD)	22.7 (14.7)$_a$	16.0 (9.1)$_{a,b}$	15.5 (7.9)$_{a,b}$	12.0 (7.5)$_b$	0.035*

Table 2

Regression data for Age and PMI vs. urate and precursors

Xanthine levels correlated with both age and PMI ($p<0.05$), whereas they do not predict urate or other precursor levels (PMI vs adenosine appears significant, but values are at limit of detection and violate homoscedasticity assumptions).

Regression data	Age			PMI		
	β	t	P	β	t	P
Urate	0.114	1.528	0.128	0.042	0.560	0.576
Xanthine	−0.172	−2.323	0.021*	0.243	3.349	0.001*
Hypoxanthine	−0.053	−0.713	0.477	0.137	1.845	0.067
Inosine	−0.026	−0.344	0.731	−0.135	−1.816	0.071
Adenosine	0.039	0.519	0.604	−0.235	−3.227	0.001*

Appendix F

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Neuroscience 2013

Presentation Abstract

Program#/Poster#:	714.09/H15
Presentation Title:	Astroglia-dependent protective mechanisms of urate in a cellular model of Parkinson's disease
Location:	Halls B-H
Presentation time:	Wednesday, Nov 13, 2013, 8:00 AM - 9:00 AM
Topic:	++C.04.i. Cellular mechanisms
Authors:	***R. BAKSHI**, M. MAGUIRE, R. LOGAN, X. CHEN, M. A. SCHWARZSCHILD; Neurol., Massachusetts Gen. Hosp., Boston, MA
Abstract:	Urate has emerged as a promising candidate therapeutic target for people with Parkinson's disease (PD) based on its antioxidant and neuroprotective properties, and on its identification as a predictor of a reduced PD risk and a milder rate of disease progression. We recently reported that urate produced much of its protective effect indirectly via astroglial cells. To further characterize the mechanisms underlying the astrocyte-dependence of urate's neuroprotection in cellular models of PD, we have employed complementary biochemical techniques and targeted screens to identify the putative protective factor(s) released by urate-stimulated astrocytes. In these studies, we have treated enriched astroglial cultures with varying concentrations of urate, or vehicle followed by cell viability evaluation and FACS analysis with Annexin/PI staining. We confirmed a significant protective effect of conditioned medium from urate-treated astrocytes on MES 23.5 cell viability exposed to 200 μM H_2O_2. From our targeted screens, glutathione (GSH), which is detected at higher concentrations in the conditioned medium of the urate-treated (compared to vehicle-treated) astrocytes, emerged as a primary candidate for the putative neuroprotective factor. Intracellular levels of GSH were also significantly increased in urate-treated astrocytes compared to controls. We blocked GSH function in the conditioned media by targeting its synthesis or its uptake by neuronal cells by using L-buthionine-sulfoximine

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(BSO), which inhibits GSH synthesis thereby reducing cellular GSH levels, or Acivicin, which inhibits g-glutamyl transpeptidase and transmembrane glutathione transport, respectively. These inhibition assays showed that loss of GSH function in the conditioned media attenuates urate's protection of neuronal cells from H_2O_2 toxicity. Overall, these results implicate GSH as the astrocytic protective factor mediating the urate's protective effect in this cellular model of PD.

Disclosures: **R. Bakshi:** None. **M. Maguire:** None. **R. Logan:** None. **X. Chen:** None. **M.A. Schwarzschild:** None.

Keyword(s): URATE

PARKINSON'S DISEASE

GLUTATHIONE

Support: Dept of Defense/NETPR program W81XWH-11-1-0150

NIH K24NS060991

Appendix G

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Neuroscience 2013

Presentation Abstract

Program#/Poster#:	329.20/I18
Presentation Title:	Urate oxidase knockout in mice by inducible cre and their cns phenotype
Location:	Halls B-H
Presentation time:	Monday, Nov 11, 2013, 11:00 AM -12:00 PM
Topic:	++C.04.d. Transgenic and related mouse models
Authors:	***F. ZUO**, M. MAGUIRE, R. LOGAN, Y. XU, X. CHEN, M. SCHWARZSCHILD; MGH Mol. Neurobio. Lab., Charlestown, MA
Abstract:	Oxidative stress has been implicated as a core contributor to the neurodegenerative process in Parkinson's disease (PD). Levels of urate, a natural antioxidant are inversely associated with both the risk and progression of PD, prompting clinical development of urate elevation as a potential treatment for PD. In mice, functional urate oxidase (UOx, which is absent in humans) catalyzes the metabolism of urate to allantoin. We previously reported successful manipulation of CNS urate by complementary genetic approaches that attenuated or exacerbated parkinsonain 6-hydroxydopamine-induced neuroxicity in *UOx* global knockout (KO) mice and transgenic (Tg) *UOx* mice, respectively. The confounding developmental effects of constitutive *UOx* disruption including renal pathology in *UOx* global KO mice limits their utility for further study of urate in neurobiology and PD models. We therefore developed $UOx^{flox/flox}$ mice through a commercial service by floxing a 775 bp region including exons 3-4 in *UOx*. $UOx^{flox/flox}$ mice were mated with Tg *UBC-cre* mice (obtained from the Jackson Laboratory) to generate *UBC-cre* $UOx^{flox/+}$ mice. *UBC-cre* $UOx^{flox/flox}$ mice and their non-Tg (NTg) littermate controls (NTg $UOx^{flox/flox}$ mice) were then generated through $UOx^{flox/flox}$ and *UBC-cre* $UOx^{flox/+}$ mating. Recombination was induced by 75 mg/kg tamoxifen i.p.. Liver UOx protein in *UBC-cre* $UOx^{flox/flox}$ mice was significantly lower at 7 and 28 days after completing

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tamoxifen administration, suggesting successful induction of Cre-catalyzed recombination and KO of *UOx*. High performance liquid chromatography coupled to electrochemical detection of urate levels demonstrated strong trends towards increased urate in both blood and brain (striatum) in *UBC-cre* $UOx^{flox/flox}$ mice at 7 and 28 days after tamoxifen treatment as compared with NTg $UOx^{flox/flox}$ littermates. We will further characterize these *UOx* conditional KO mice and their CNS phenotypes in models of PD.
Supported by: the RJG Foundation, Michael J. Fox Foundation, American Federation for Aging Research Beeson Collaborative Program, R21NS058324, K24NS060991 and DoD W81XWH-11-1-0150.

Disclosures: **F. Zuo:** None. **M. Maguire:** None. **R. Logan:** None. **Y. Xu:** None. **X. Chen:** None. **M. Schwarzschild:** None.

Keyword(s): URATE OXIDASE

KNOCKOUT MICE

Support: RJG Foundation R21NS058324

Michael J. Fox Foundation K24NS060991

American Federation for Aging Research Beeson Collaborative Program DoD W81XWH-11-1-0150